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<b>(21) International Application Number:</b> PCT/NL99/00367 <b>(22) International Filing Date:</b> 11 June 1999 (11.06.99) <b>(30) Priority Data:</b> 09/097,239 12 June 1998 (12.06.98) US <b>(71) Applicant:</b> INTROGENE B.V. [NL/NL]; Wassenaarseweg 72, NL-2333 AL Leiden (NL). <b>(72) Inventors:</b> SCHOUTEN, Govert; Da Costastraat 82, NL-2321 AR Leiden (NL). VOGELS, Ronald; Van Rietlaan 4, NL-3461 HW Linschoten (NL). BOUT, Abraham; Coymansstraat 24, NL-2751 AR Moerkapelle (NL). VAN ES, Helmuth; Bandholm 89, NL-2133 DJ Hoofddorp (NL). <b>(74) Agent:</b> OTTEVANGERS, S., U.; Verenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).		<b>(81) Designated States:</b> AU, CA, JP, MX, NZ.  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> HIGH-THROUGHPUT SCREENING OF GENE FUNCTION USING LIBRARIES FOR FUNCTIONAL GENOMICS APPLICATIONS  <p style="text-align: center;">Construction total Adeno cDNA Library (II)</p>		
<b>(57) Abstract</b> <p>Novel means and methods for their use are provided to determine the function of the product(s) of one or more sample nucleic acids. The sample nucleic acids are synthetic oligonucleotides, DNA, or cDNA and encode polypeptides, antisense nucleic acids or GSEs. The sample nucleic acids are expressed in a host by a vehicle to alter at least one phenotype of the host. The altered phenotype(s) is identified as a means to assign a biological function to the product(s) encoded by the sample nucleic acid(s).</p>		

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Title: High-throughput screening of gene function using libraries for functional genomics applications.

The invention is related to high-throughput methods for identifying the function of sample nucleic acids and their products. The invention is exemplified by the use of the E1-complementing adenoviral packaging cell line PER.C6 in combination with an E1-deleted plasmid-based generation system to produce recombinant adenovirus vectors in a high throughput setting to functionate the product of a sample nucleic acid.

The ultimate goal of the Human Genome Project is to sequence the entire human genome. The expected outcome of this effort is a precise map of the 70,000-100,000 genes that are expressed in man. However, a fairly complete inventory of human coding sequences will most likely be publicly available sooner. Since the early 1980s, a large number of Expressed Sequence Tags (ESTs; partial DNA sequences read from the ends of cDNA molecules) have been obtained by both government and private research organizations. A hallmark of these endeavors, carried out by a collaboration between Washington University Genome Sequencing Center and members of the IMAGE (Integrated Molecular Analysis of Gene Expression) consortium (<http://www-bio.llnl.gov/bbrp/image/image.html>), has been the rapid deposition of the sequences into the public domain and the concomitant availability of the sequence-tagged cDNA clones from several distributors (Marra, et al. (1998) *Trends Genet.* 14 (1):4-7). At present, the collection of cDNAs is believed to represent approximately 50,000 different human genes expressed in a variety of tissues including liver, brain, spleen, B-cells, kidney, muscle, heart, alimentary tract, retina, hypothalamus, and the number is growing daily.

Recent initiatives like that of the Cancer Genome Anatomy project support an effort to obtain full-length sequences of clones in the Unigene set (a set of cDNA clones

that is publicly available) by the year 1999. At the same time, commercial entities propose to validate (re-sequence) 40,000 full-length cDNA clones by 1999 and the individual clones will be available to any interested party. The speed  
5 by which the coding sequences of novel genes are identified is in sharp contrast to the rate by which the function of these genes is elucidated. Assigning functions to the cDNAs in the databases, or functional genomics, is a major challenge in biotechnology today.

10 For decades, novel genes were identified as a result of research designed to explain a biological process or hereditary disease and the function of the gene preceded its identification. In functional genomics, coding sequences of genes are first cloned and sequenced and the sequences are  
15 then used to find functions. Although other organisms such as *Drosophila*, *C. elegans*, and Zebrafish are highly useful for the analysis of fundamental genes, for complex mammalian physiological traits (blood glucose, cardiovascular disease, inflammation) animal model systems are inevitable. However,  
20 the slow rate of reproduction and the high housing costs of the animal models are a major limitation to high-throughput functional analysis of genes. Although labor-intensive efforts are made to establish libraries of mouse strains with chemically or genetically mutated (tagged) genes in a search  
25 for phenotypes that allow the elucidation of gene function or that are related to human diseases, a systematic analysis of the complete spectrum of mammalian genes, be it human or animal, is a significant task.

In order to keep pace with the volume of sequence data,  
30 the field of functional genomics needs the ability to perform high-throughput analysis of true gene function. Recently, a number of techniques have been developed that are designed to link tissue and cell specific gene expression to gene function. These include cDNA microarraying and gene chip  
35 technology and differential display mRNA. Serial Analysis of Gene Expression (SAGE) or differential display of messenger



RNA can identify genes that are expressed in tumor tissue but are absent in the respective normal or healthy tissue. In this way, potential genes with regulatory functions can be selected from the excess of ubiquitously expressed genes that have a less-likely chance to be useful for small drug screening or gene therapy projects. Gene chip technology has the potential to allow the monitoring of gene expression through the measurement of mRNA expression levels in cells of a large number of genes in only a few hours. Cells cultured under a variety of conditions can be analyzed for their mRNA expression patterns and compared. Currently, DNA microarray chips with 40,000 non-redundant human genes are produced and are planned to be on the market in 1999 (Editorial (1998) *Nat. Genet.* 18(3):195-7.). However, these techniques are primarily designed for screening cancer cells and not for screening for specific gene functions.

Double or triple hybrid systems also are used to add functional data to the genomic databases. These techniques assay for protein-protein, protein-RNA, or protein-DNA interactions in yeast or mammalian cells (Brent and Finley (1997) *Annu. Rev. Genet.* 31:663-704). However, this technology does not provide a means to assay for a large number of other gene functions such as differentiation, motility, signal transduction, enzyme and transport activity. Yeast expression systems have been developed which are used to screen for naturally secreted and membrane proteins of mammalian origin (Klein, et al. (1996) *Proc. Natl. Acad. Sci. USA* 93 (14):7108-13). This system also allows for collapsing of large libraries into libraries with certain characteristics which aid in the identification of specific genes and gene products. A disadvantage of this system is that genes encoding secreted proteins primarily are selected. Secondly, this technology is based on yeast as a heterologous expression system and therefore there will be gene products that are not appropriately folded resulting in a biased library.

Other current strategies include the creation of transgenic mice or knockout mice. A successful example of gene discovery by such an approach is the identification of the osteoprotegerin gene. DNA databases were screened to  
5 select ESTs with features suggesting the cognate genes encoded secreted proteins. The biological functions of the genes were assessed by placing the corresponding full-length cDNAs under the control of a liver-specific promoter. Transgenic mice created with each of these constructs  
10 consequently have high plasma levels of the relevant protein. Subsequently, the transgenic animals were subjected to a battery of qualitative and quantitative phenotypic investigations. One of the genes that was transfected into mice produced mice with an increased bone density, which led  
15 subsequently to the discovery of a potent anti-osteoporosis factor (Simonet, et al. (1997) *Cell*. 89(2):309-19). Such a method has the disadvantages that it is costly and highly time consuming.

The challenge in functional genomics is to develop and  
20 refine all the above-described techniques and integrate their results with existing data in a well-developed database that provides for the development of a picture of how gene function constitutes cellular metabolism and a means for this knowledge to be put to use in the development of novel  
25 medicinal products. The current technologies have limitations and do not necessarily result in true functional data. Therefore, there is a need for a method that allows for direct measurement of function of a single gene from a collection of genes (gene pools or individual clones) in a  
30 high-throughput setting in appropriate *in vitro* assay systems and animal models.

The development of high throughput screens is discussed in Jayawickreme and Kost, (1997) *Curr. Opin. Biotechnol.*  
35 8:629-634. A high throughput screen for rarely transcribed differentially expressed genes is described in von Stein et

al., (1997) *Nucleic Acids Res.* 35: 2598-2602. High  
throughput genotyping is disclosed in Hall et al., (1996)  
*Genome Res.* 6:781-790. Methods for screening transdominant  
intracellular effector peptides and RNA molecules are  
5 disclosed in Nolan, WO97/27212 and WO/9727213.

Methods, and compositions for use therein, are provided  
for directly, rapidly and unambiguously measuring in a high  
throughput setting the function of sample nucleic acids of  
10 unknown function, using a plasmid-based E1-deleted adenoviral  
vector system and an E1-complementing host cell. The method  
includes the steps of constructing a set of adapter plasmids  
by inserting a set of cDNAs, DNAs, ESTs, genes, synthetic  
oligonucleotides or a library of nucleic acids into E1-  
15 deleted adapter plasmids, cotransfecting an E1-complementing  
cell line with the set or library of adapter plasmids and a  
plasmid(s) having sequences homologous to sequences in the  
set of adapter plasmids and which also includes all  
adenoviral genes not provided by the complementing cell line  
20 or adapter plasmids necessary for replication and packaging  
to produce a set or library of recombinant adenoviral vectors  
preferably in a miniaturized, high throughput setting. To  
identify and assign function to product(s) encoded by the  
sample nucleic acids, a host is transduced in a high  
25 throughput setting with the recombinant adenoviral vectors  
which express the product(s) of the sample nucleic acids and  
thereby alter a phenotype of a host. The altered phenotype  
is identified and used as the basis to assign a function to  
the product(s) encoded by the sample nucleic acids. The  
30 plasmid-based system is used to rapidly produce adenovirus  
vector libraries that are preferably RCA-free for high  
throughput screening. Each step of the method can be  
performed in a multiwell format and automated to further  
increase the capacity of the system. This high throughput  
35 system facilitates expression analysis of a large number of  
sample nucleic acids from human and other organisms both in

*vitro* and *in vivo* and is a significant improvement over other available techniques in the field.

#### BRIEF DESCRIPTION OF THE DRAWINGS

5        Figure 1: Construction of pBS.PGK.PCRI. pBS.PGK.PCRI encodes the human phosphoglycerate kinase promoter (PGK) operatively linked to adenovirus 5 (Ad5) E1 nucleotides 459-916. To construct this plasmid, Ad5 nucleotides 459-916 were PCR amplified with primers Ea-1 (SEQ ID NO:27) and Ea-2 (SEQ  
10 ID NO:28), digested with *Cla*I and cloned into the *Cla*I-*Eco*RV sites of pBluescript (Stratagene), resulting in pBS.PCRI. The PGK promoter was excised from pTN by complete digestion with *Sca*I and partial digestion with *Eco*RI and cloned into the corresponding sites of pBS.PCRI, resulting in  
15 pBS.PGK.PCRI.

      Figure 2: Construction of pIG.E1A.E1B.X. pIG.E1A.E1B.X encodes Ad5 nucleotides 459-5788 (E1A and E1B regions) operatively linked to the human PGK promoter. pIG.E1A.E1B.X also encodes Ad5 pIX protein. pIG.E1A.E1B.X was constructed  
20 by replacing the *Sca*I-*Bsp*EI fragment of pAT-X/S with the corresponding fragment of pBS.PGK.PCRI.

      Figure 3A: Construction of pAT-PCR2-NEO. To construct this plasmid, the E1B promoter and initiation codon (ATG) of the E1B 21kDa protein were PCR amplified with primers Ea-3  
25 (SEQ ID NO:29) and Ep-2 (SEQ ID NO:30), where Ep-2 introduces an *Nco*I site (5'-CCATGG) at the 21kDa protein initiation codon. The PCR product (PCRII) was digested with *Hpa*I and *Nco*I and ligated into the corresponding sites of pAT-X/S, producing pAT-X/S-PCR2. The *Nco*I-*Stu*I fragment of pTN,  
30 containing the Neo<sup>R</sup> and a portion of the HBV poly(A) site were ligated into the *Nco*I-*Nru*I sites of pAT-X/S-PCR2, producing pAT-PCR2-NEO.

      Figure 3B: Construction of pIG.E1A.NEO. pIG.E1A.NEO encodes Ad5 nucleotides 459-1713 operatively linked to the  
35 human PGK promoter. Also encoded is the E1B promoter

functionally linked to the neomycin resistance gene (Neo<sup>R</sup>) and the hepatitis B virus (HBV) poly(A) signal. In this construct, the AUG codon of the E1B 21 kDa protein functions as the initiation codon of Neo<sup>R</sup>. The HBV poly(A) signal of pAT-PCR2-NEO (see Fig. 3A) was completed by replacing the *ScaI*-*SalI* fragment of pAT-PCR2-NEO with the corresponding fragment of pTN, producing pAT.PCR2.NEO.p(A), and replacing the *ScaI*-*XbaI* fragment of pAT.PCR2.NEO.p(A) with the corresponding fragment of pIG.E1A.E1B.X, producing pIG.E1A.NEO.

Figure 4: Construction of pIG.E1A.E1B. pIG.E1A.E1B contains the Ad5 nucleotides 459-3510 (E1A and E1B proteins) operatively linked to the PGK promoter and HBV poly(A) signal. This plasmid was constructed by PCR amplification of the N-terminal amino acids of the E1B 55 kD protein with primers Eb-1 (SEQ ID NO:31) and Eb-2 (SEQ ID NO:32), which introduces an *XhoI* site, digested with *BglIII* and cloned into the *BglIII*-*NruI* sites of pAT-X/S, producing pAT-PCR3. The *XbaI*-*XhoI* fragment of pAT-PCR3 was replaced with the *XbaI*-*SalI* fragment (containing the HBV poly(A) site) of pIG.E1A.NEO to produce pIG.E1A.E1B.

Figure 5: Construction of pIG.NEO. pIG.NEO contains the Neo<sup>R</sup> operatively linked to the E1B promoter. pIG.NEO was constructed by ligating the *HpaI*-*ScaI* fragment of pIG.E1A.NEO which contains the E1B promoter and Neo<sup>R</sup> into the *EcoRV*-*ScaI* sites of pBS.

Figure 6: Transformation of primary baby rat kidney (BRK) cells by adenovirus packaging constructs. Subconfluent dishes of BRK cells were transfected with 1 or 5 µg of either pIG.NEO, pIG.E1A.NEO, pIG.E1A.E1B, pIG.E1A.E1B.X, pAd5XhoIC, or pIG.E1A.NEO plus pDC26, which expresses the Ad5 E1B gene under control of the SV40 early promoter. Three weeks post-transfection, foci were visible, cells were fixed, Giemsa stained and the foci counted. The results shown are the average number of foci per 5 replicate dishes.

Figure 7: Western blot analysis of A549 clones transfected with pIG.E1A.NEO and human embryonic retinoblasts (HER cells) transfected with pIG.E1A.E1B (PER clones). Expression of Ad5 E1A and E1B 55 kD and 21 kD proteins in transfected A549 cells and PER cells was determined by Western blot with mouse monoclonal antibodies (Mab) M73 which recognizes E1A gene products and Mabs AIC6 and C1G11, which recognize the E1B 55 kDa and 21 kDa proteins, respectively. Mab binding was visualized using horseradish peroxidase-labeled goat anti-mouse antibody and enhanced chemiluminescence. 293 and 911 cells served as controls.

Figure 8: Southern blot analysis of 293, 911 and PER cell lines. Cellular DNA was extracted, *HindIII* digested, electrophoresed and transferred to Hybond N+ membranes (Amersham). Membranes were hybridized to radiolabeled probes generated by random priming of the *SspI-HindIII* fragment of pAd5.SalB (Ad5 nucleotides 342-2805).

Figure 9: Transfection efficiency of PER.C3, PER.C5, PER.C6 and 911 cells. Cells were cultured in 6-well plates and transfected in duplicate with 5  $\mu$ g pRSV.lacZ by calcium-phosphate co-precipitation. Forty-eight hours post-transfection, cells were stained with X-Gal and blue cells were counted. Results shown are the mean percentage of blue cells per well.

Figure 10: Construction of adenovirus vector, pMLPI.TK. pMLPI.TK was designed to have no sequence overlap with the packaging construct pIG.E1A.E1B. pMLPI.TK was derived from pMLP.TK by deletion of the region of sequence overlap with pIG.E1A.E1B and deletion of non-coding sequences derived from lacZ. SV40 poly(A) sequences of pMLP.TK were PCR amplified with primers SV40-1 (SEQ ID NO:33), which introduces a *BamHI* site and SV40-2 (SEQ ID NO:34), which introduces a *BglII* site. pMLP.TK Ad5 sequences 2496 to 2779 were PCR amplified with primers Ad5-1 (SEQ ID NO:35), which introduces a *BglII* site and Ad5-2 (SEQ ID NO:36). Both PCR products were *BglII* digested, ligated, and PCR amplified with primers SV40-1 and

Ad5-2. This third PCR product was *Bam*HI and *Afl*III digested and ligated into the corresponding sites of pMLP.TK, producing pMLPI.TK.

Figure 11A: New adenovirus packaging construct, pIG.E1A.E1B, does not have sequence overlap with new adenovirus vector, pMLPI.TK. Regions of sequence overlap between the packaging construct, pAd5XhoIC expressed in 911 cells and adenovirus vector, pMLP.TK, that can result in homologous recombination and the formation of replication competent adenovirus are shown. In contrast, there are no regions of sequence overlap between the new packaging construct, pIG.E1A.E1B, expressed in PER.C6 cells, and the new adenovirus vector, pMLPI.TK.

Figure 11B: New adenovirus packaging construct, pIG.E1A.NEO, does not have sequence overlap with new adenovirus vector, pMLPI.TK. There are no region of sequence overlap between the new packaging construct, pIG.E1A.NEO and the new adenovirus vector, pMLPI.TK, that can result in homologous recombination and the formation of replication competent adenovirus.

Figure 12: Generation of recombinant adenovirus, IG.Ad.MLPI.TK. Recombinant adenovirus, IG.Ad.MLPI.TK, was generated by co-transfection of 293 cells, with *Sal*I linearized pMLPI.TK and the right arm of *Cla*I digested, wild-type Ad5 DNA. Homologous recombination between linearized pMLPI.TK and wild-type Ad5 DNA produces IG.Ad.MLPI.TK DNA, which contains an E1 deletion of nucleotides 459-3510. 293 cells transcomplement the deleted Ad5 genome, thereby, permitting replication of the IG.Ad.MLPI.TK DNA and its packaging into virus particles.

Figure 13: Rationale for the design of adenovirus-derived recombinant DNA molecules that duplicate and replicate in cells expressing adenovirus replication proteins. A diagram of the adenovirus double-stranded DNA genome indicating the approximate locations of E1, E2, E3, E4, and L regions is shown. The terminal polypeptide (TP)

attached to the 5'-termini is indicated by closed circles. The right arm of the adenovirus genome can be purified by removal of the left arm by restriction enzyme digestion.

Following transfection of the right arm into 293 or 911 cells, adenoviral DNA polymerase (white arrow) encoded on the right arm, will produce only single-stranded forms. Neither the double-stranded or single-stranded DNA can replicate because they lack an ITR at one termini. Providing the single-stranded DNA with a sequence that can form a hairpin structure at the 3'-terminus that can serve as a substrate for DNA polymerase will extend the hairpin structure along the entire length of the molecule. This molecule can also serve as a substrate for a DNA polymerase but the product is a duplicated molecule with ITRs at both termini that can replicate in the presence of adenoviral proteins.

Figure 14: Adenovirus genome replication. The adenovirus genome is shown in the top left. The origins of replication are located within the left and right ITRs at the genome ends. DNA replication occurs in two stages.

Replication proceeds from one ITR generating a daughter duplex and a displaced parental single-strand which is coated with adenovirus DNA binding protein (DBP, open circles) and can form a panhandle structure by annealing of the ITR sequences at both termini. The panhandle is a substrate for DNA polymerase (Pol: white arrows) to produce double-stranded genomic DNA. Alternatively, replication proceeds from both ITRs, generating two daughter molecules, thereby, obviating the requirement for a panhandle structure.

Figure 15: Potential hairpin conformation of a single-stranded DNA molecule that contains the HP/asp sequence (SEQ ID NO:47). Asp718I digestion of pICLha, containing the cloned oligonucleotides, HP/asp1 and HP/asp2 yields a linear double-stranded DNA with an Ad5 ITR at one terminus and the HP/asp sequence at the other terminus. In cells, expressing the adenovirus E2 region, a single-stranded DNA is produced with an Ad5 ITR at the 5'-terminus and the hairpin



conformation at the 3'-terminus. Once formed, the hairpin can serve as a primer for cellular and/or adenovirus DNA polymerase to convert the single stranded DNA to double stranded DNA.

5       Figure 16: Diagram of pICLhac. pICLhac contains all the elements of pICL (Figure 19) but also contains in the Asp718 site, the HP/asp sequence in an orientation that will produce the hairpin structure shown in Figure 15, following linearization by Asp718 digestion and transfection into cells  
10       expressing adenovirus E2 proteins.

      Figure 17: Diagram of pICLhaw. pICLhaw is identical to pICLhac (Figure 16) with the exception that the inserted HP/asp sequence is in the opposite orientation.

      Figure 18: Schematic representation of pICLI. pICLI  
15       contains all the elements of pICL (Figure 19) but also contains in the Asp718 site, an Ad5 ITR.

      Figure 19: Diagram of pICL. pICL is derived from the following: (i) nucleotides 1-457, Ad5 nucleotides 1-457 including the left ITR, (ii) nucleotides 458-969, human CMV  
20       enhancer and immediate early promoter, (iii) nucleotides 970-1204, SV40 19S exon and truncated 16/19S intron, (iv) nucleotides 1218-2987, firefly luciferase gene, (v) nucleotides 3018-3131, SV40 tandem polyadenylation signals from the late transcript, (vi) nucleotides 3132-5620, pUC12  
25       sequences including an Asp718 site, and (vii) ampicillin resistance gene in reverse orientation.

      Figure 20: Shows a schematic overview of the adenovirus fragments cloned in pBr322 (plasmid) or pWE15 (cosmid) derived vectors. The top line depicts the complete adenovirus  
30       genome flanked by its ITRs (filled rectangles) and with some restriction sites indicated. Numbers following restriction sites indicate approximate digestion sites (in kb) in the Ad5 genome.

      Figure 21: Drawing of adapter plasmid pAd/L420-HSA

35       Figure 22: Drawing of adapter plasmid pAd/Clip

Figure 23: Schematic presentation of the generation of recombinant adenoviruses using a plasmid-based system. In the top the genome organization of Ad5 is given with filled boxes representing the different early and late transcription regions, and flanking ITRs. The middle presents the two DNAs used for a single homologous recombination and, after transfection into packaging cells, leading to the recombinant virus (represented at the bottom).

Figure 24: Drawing of minimal adenoviral vector pMV/L420H

Figure 25: Helper construct for replication and packaging of minimal adenoviral vectors. Schematic presentation of the cloning steps for the generation of the helper construct pWE/AdΔ5'.

Figure 26: Evidence for SV40-LargeT/ori mediated replication of large adenoviral constructs in COS-1 cells. Figure 26A shows a schematic presentation of construct pWE/Ad.Δ5'. The location of the SV40 ori sequence and the fragments used to prepare probes are indicated. Evidence for SV40-LargeT/ori mediated replication of large adenoviral constructs in COS-1 cells. Figure 26B shows an autoradiogram of the Southern blot hybridized to the adenovirus probe. Figure 26C shows an autoradiogram of the Southern blot hybridized to the pWE probe. Lanes 1, marker lane: λ DNA digested with *EcoRI* and *HindIII*. Lane 4 is empty. Lanes 2, 5, 7, 9, 11, 13, 15 and 17 contain undigested DNA and Lanes 3, 6, 8, 10, 12, 14, 16 and 18 contain *MboI* digested DNA. All lanes contain DNA from COS-1 cells as described in the text transfected with pWE.pac (lanes 2 and 3), pWE/Ad.Δ5' construct #1 (lanes 5 and 6), #5 (lanes 7 and 8) and #9 (lanes 9 and 10), pWE/Ad.AflII-rITR (lanes 11 and 12), pMV/CMV-LacZ (lanes 13 and 14), pWE.pac digested with *PacI* (lanes 15 and 16) or pWE/Ad.AflII-rITR digested with *PacI* (lanes 17 and 18). Arrows point at the expected positive signal of 1416 bp (Figure 26B) and 887 bp (Figure 26C).

Figure 27. Production of E1/E2A deleted adenoviral vectors and its efficiency in miniaturized PER.C6/E2A based production system (example 10).

5 Figure 28. Average titers produced in a 96 well plate as measured using a PER.C6/E2A based plaque assay (example 11).

Figure 29. Fidelity of adenoviral vector production miniaturized PER.C6/E2A based production system for a number of marker and human cDNA transgenes (example 12).

10 Figure 30: Relative amounts of wells with CPE after transfection of PER.C6/E2A cells with pCLIP-LacZ, purified by 6 different protocols (example 13). Qiagen: standard alkaline lysis followed by Qiagen plasmid purification; AlkLys: alkaline lysis followed by isopropanol precipitation, and solubilization in TE buffer; AL + RNase: alkaline lysis  
15 followed by isopropanol precipitation, and solubilization in TE buffer containing RNase at 10 microgram per ml; AL+R+phenol: alkaline lysis followed by isopropanol precipitation, and solubilization in TE buffer containing RNase at 10 microgram per ml, followed by phenol/chloroform  
20 extraction and ethanol precipitation; CTAB: Standard CTAB plasmid isolation; CTAB+phenol: Standard CTAB plasmid isolation, but solubilization in TE buffer containing RNase at 10 microgram per ml, followed by phenol/chloroform extraction.

25 Figure 31. Effect of using digested plasmid for transfection without phenol-chloroform cleaning (example 14). The results of all experiments are depicted and expressed as percentage of wells showing CPE formation. A) LacZ-adapter DNA was isolated using 6 different isolation methods (see  
30 example 13); 1: Qiagen, 2: Alkaline lysis, 3: Alkaline lysis + RNase treatment, 4: Alkaline lysis + RNase treatment + p/c purification of DNA before linerization, 5: CTAB (cetyltrimethylammoniumbromide), 6: CTAB + p/c purification of DNA before linerization, rITR was p/c purified, B)  
35 Purified and unpurified EGFP- and EYFP-adapter DNA, rITR was p/c purified, C) EGFP-adapter DNA and rITR were tested

purified and unpurified; 1: Both adapter and rITR purified (control), 2: rITR purified, adapter DNA unpurified, 3: rITR and adapter unpurified.

Figure 32. Stability of adenoviral vectors produced in miniaturized format and incubated for up to three weeks at three different temperatures and measured using a plaque forming assay for adenoviral vectors (example 15).

Figure 33. Efficiencies of virus generation in percentages of CPE after virus generation of several adenoviruses (E1 and E2A deleted) containing cDNAs in antisense (AS) orientation (example 16).

Figure 34A-M. Plasmid maps of adenoviral adapter plasmid (example 17). These adenoviral adapter plasmids are particularly useful for the construction of expression libraries in adenoviral vectors such as the subject of this application. They have very rare restriction sites for the linearization of adapters and libraries of adapters (with transgenes inserted) and will not inactivate the adapter by digestion of the inserts.

Figure 34M: The cosmid containing pIPspAdapt5- or pCLIP-IppoI-polynew-derived adenoviral DNA can be used for *in vitro* ligations. Double stranded oligonucleotides containing compatible overhangs are ligated between the I-CeuI and PI-SceI sites, between I-CeuI and I-PpoI, between I-SceI and PI-SceI, and between I-SceI and I-PpoI. The PacI restriction endonuclease is subsequently used not only to linearize the construct after ligation and thereby to liberate the left- and right ITR, but also to eliminate non-recombinants.

Figure 34N. Relative amounts of wells with CPE after transfection of PER.C6/E2A cells with pCLIP-LacZ and the adapter plasmid pIPspAdapt2.

Figure 35. (example 19). Percentage of virus producing wells (CPE positive) in a 96-well plate of PER.C6/E2A cell after propagation of the freeze/thawed transfected cell lysates. Helper molecules used for cotransfection were (1)

pWE/Ad.AflIII-rITRsp, (2) pWE/Ad.AflIII-rITRsp.dE2A, (3) pWE/Ad.AflIII-rITRsp.dXba, and (4) pWE/Ad.AflIII-rITR.

Figure 36 (I and II) (example 20). Schematic overview of constructing an arrayed adenoviral cDNA expression library.

5        Figure 37 (example 21). Comparison of co-transfections of different adapter plasmids and pWE/Ad.AflIII-rITRDE2A on 384 well plates with co-transfections on 96 well plates. Shown is the percentage of virus producing wells (CPE positive wells) scored at different time points as indicated after propagation of the freeze/thawed transfected cells to  
10       new PER.C6/E2A cells 5 days after transfection (upper panel) or 7 days after transfection (lower panel).

Figure 38A,B,C (example 22). The percentage of virus producing wells (CPE positive wells) scored at different time  
15       points as indicated after changing the medium of the transfected cells 7 days after transfection (A); after no medium change (B); and after standard propagation of the freeze/thawed transfected cells to new PER.C6/E2A cells.

Figure 39 (example 23). The percentage of virus  
20       producing cells (CPE positive) wells scored after propagation of the freeze/thawed transfected cells to new PER.C6/E2A cells, in three different experiments using PER.C6/E2A cells for transfections with indicated confluency at time of transfection. The figure legend refers to table 9 where the  
25       absolute cell numbers from each flask in each experiment were counted. The cells from these flasks were used to seed 96 well plates for transfection with adenoviral adapter and helper DNA molecules.

Figure 40. The use of adenoviral expression vectors as a  
30       semi-stable expression system for assays with a delayed readout of phenotype after infection with an adenoviral expression library (example 24). Transgene used: Green Fluorescent Protein (EGFP, Clontech). A crude PER.C6/E2A production lysate was used at an MOI of about 500-1000.

35       Figure 41. The use of PEI for generating adenoviral vectors in miniaturized format (example 25). Transfection

efficiency, virus formation (CPE) and proliferation (toxicity) are depicted.

Figure 42. Effect of temperature PEI at time of transfections on CPE efficiency (example 25). W: Warm (room  
5 temperature) and C: Cold (4 °C).

Figure 43. Effect of PEI transfection volume on transfection efficiencies (example 25).

Figure 44. Washing of PER.C6/E2A cells with serum free medium before applying lipofectamine-DNA complex can be  
10 omitted (example 26).

In one aspect the invention provides a library of expressible nucleic acids comprising a multiplicity of compartments, each comprising at least one vehicle comprising  
15 at least one nucleic acid of said library, whereby said vehicle is capable of very efficiently introducing said at least one nucleic acid in a cell such that it can be expressed. One advantage of said library is that said library may be introduced into cells very efficiently. Another  
20 advantage of said library is that it comprises a multiplicity of compartments each comprising at least one nucleic acid. Said library may be favourably used to study the effect of expressed nucleic acid in a cell. A library with this architecture may be favourably used to rapidly select those  
25 compartments comprising at least one nucleic acid which when expressed in a cells exerts a certain effect. When a compartment comprises only one nucleic acid then it is known that that nucleic acid exerts the effect. When a compartment comprises more than one nucleic acid then it is known that at  
30 least one of said more than one nucleic acid exerts the effect. The advantage of knowing which compartment comprises nucleic acid which can exert a certain effect is greater when said compartment comprises relatively few different nucleic acid and is highest when said compartment comprises only one  
35 nucleic acid. It is also of advantage to precisely know the

number of different nucleic acid per compartment, particularly in larger libraries.

An expressible nucleic may be any expressible nucleic acid such as a nucleic acid coding for a proteinaceous molecule, an RNA molecule or a DNA molecule.

In a preferred embodiment said vehicle comprises a viral element or a functional part, derivative and/or analogue thereof. A viral element may comprise a virus particle such as but not limited to an enveloped retrovirus particle or a virus capsid of a non-enveloped virus such as but not limited to an adenovirus. A virus particle is favourable since it allows the easy efficient introduction of said at least one nucleic acid into a cell. A viral element may also comprise a viral nucleic acid allowing the amplification of said library in cells. A viral element may comprise a viral nucleic acid allowing the packaging of said at least one nucleic acid into a vehicle when said vehicle is a virus particle.

In a preferred embodiment said viral element is derived from an adenovirus. Preferably said vehicle comprises an adenovirus vector packaged into an adenovirus capsid.

A cell may be any kind of cell. For instance when said library is screened for the presence of nucleic acid with potential therapeutic value said cell preferably is a eukaryotic cell, preferably a mammalian cell.

In one embodiment at least one compartment comprises at least two of said at least one vehicle. Especially but not limited to large libraries it becomes advantageous to reduce the number of compartments to reduce at least in part the number of screening assays that need to be performed. For example in such cases libraries may be provided that comprise more than one vehicle. When subsequent to a screening assay a certain effect is correlated to a certain compartment said vehicles in said compartment may be analysed separately in an additional screening assay to select the vehicle comprising nucleic acid the expression of which exerts the effect. One the other hand however, more than one vehicle present in a

compartment may be favourably used in another setting. For instance when a library containing one vehicle per compartment is screened for nucleic acid capable of exerting an effect in combination with one particular other nucleic acid. Said other nucleic acid may then be provided to said cell through adding a vehicle comprising said particular other nucleic acid to all compartments prior to performing the screening assay. Similarly said at least one vehicle may comprise at least two nucleic acids.

10 In a preferred embodiment said nucleic acid derived from an adenovirus comprises nucleic acid encoding an adenovirus late protein or a functional part, derivative and/or analogue thereof. An adenovirus late protein for instance an adenovirus fiber protein may be favourably used to target said at least one vehicle to a certain cell or induce enhanced delivery of said at least one vehicle to said cell. Preferably said nucleic acid derived from an adenovirus encodes for essentially all adenovirus late proteins enabling the formation of entire adenovirus capsids, or functional parts, analogues and/or derivatives thereof. Preferably said nucleic acid derived from an adenovirus comprises nucleic acid encoding adenovirus E2A or a functional part, derivative and/or analogue thereof. Preferably nucleic acid derived from an adenovirus comprises nucleic acid encoding at least one E4-region protein or a functional part, derivative and/or analogue thereof. Thus facilitating at least in part replication of an adenovirus derived nucleic acid in a cell.

In one embodiment said nucleic acid derived from an adenovirus comprises nucleic acid encoding at least one E1-region protein or a functional part, derivative and/or analogue thereof. The presence of adenovirus nucleic acid encoding an E1-region protein facilitates at least in part replication of said nucleic acid in a cell. Such replication capacity is favoured in certain applications for instance when screening is done for expressible nucleic acid capable of irradiating tumour cells. In such cases replication of an



adenovirus nucleic acid leading to the amplification of said vehicle for instance in a mammal comprising tumour cells may lead to the irradiation of also metastasised tumour cells. On the other hand the presence of adenovirus nucleic acid  
5 encoding an E1-region protein may for instance facilitate at least in part amplification of said nucleic acid in a cell, for instance for the amplification of vehicles comprising said adenovirus nucleic acid.

In one embodiment said vehicle further comprises  
10 nucleic acid comprising an adeno-associated virus terminal repeat or a functional part, derivative and/or analogue thereof. Thus allowing the integration of said at least one nucleic acid in a cell.

In one embodiment said viral element derived from an  
15 adenovirus comprises an adenovirus capsid or a functional part, derivative and/or analogue thereof. Adenovirus biology is comparatively well known also on the molecular level. Many tools for adenovirus vectors have been and are continuing to be developed thus making an adenovirus capsid a preferred  
20 vehicle of choice for incorporating in a library of the invention. Adenovirus is capable of infecting a wide variety of cells. However, different adenovirus serotypes have different preferences for cells. To combine and widen the target cell population that an adenovirus capsid of the  
25 invention can enter in a preferred embodiment said vehicle comprises adenovirus fiber proteins from at least two adenoviruses.

In another aspect the invention provides a method for  
30 determining at least one function of at least one nucleic acid present in a library according to the invention, comprising transducing a multiplicity of cells with at least one vehicle comprising at least one nucleic acid from said library, culturing said cell while allowing for expression of  
35 said at least one nucleic acid and determining the expressed function. At present increasingly more and more nucleic acid

is sequenced and cloned. In fact cloning and sequencing of nucleic acid proceeds with such a rate that of most of the newly cloned and sequenced nucleic acid the function is not known. Also of nucleic acid with a known function, not all  
5 functions are known. It is one object of the invention to provide a method for determining function of a nucleic acid. In one aspect the invention therefore provides a method for screening a library of the invention is a screenings assay wherein a function of a nucleic acid can be assessed. In such  
10 an assay the function is central. And a library of the invention is screened for the presence of expressible nucleic acid capable of influencing at least in part said function.

In a preferred embodiment said multiplicity of cells is divided over a number of compartments each comprising at  
15 least one vehicle comprising at least one nucleic acid from said library. Said number of compartments preferably corresponds to said multiplicity of compartments of said library. In a preferred embodiment said method further comprises selecting the vehicle comprising a desired  
20 function.

In another aspect the invention provides a method for obtaining an expressible nucleic acid having a desired function when expressed in a cell comprising determining at least one function of at least one nucleic acid present in a  
25 library according to the invention, said method comprising transducing a multiplicity of cells with at least one vehicle comprising at least one nucleic acid from said library, culturing said cell while allowing for expression of said at least one nucleic acid and determining the expressed  
30 function.

In another aspect the invention provides a method for producing a library comprising a multiplicity of compartments each comprising at least one nucleic acid delivery vehicle  
35 each comprising at least one nucleic acid, said method comprising recombining vehicle nucleic acid with said at

least one nucleic acid, thereby producing a vehicle capable of delivering said at least one nucleic acid to a cell in an expressible manner. For expression of a nucleic acid a number of molecular elements well known in the field are required  
5 and/or may be used such as but not limited to promoters, enhancers, poly-adenylation signals, translation start and stop signals etc.

Said recombining may be performed through any means such as through means of molecular cloning and/or polymerase  
10 mediated amplification techniques such as PCR and NASBA. However, said recombining preferably comprises homologous recombination between at least partially overlapping sequences in vehicle nucleic acid and said at least one nucleic acid. Especially for the generation of large viral  
15 derived nucleic acid homologous recombination is preferred. Preferably said vehicle nucleic acid and/or said at least one nucleic acid comprises adenovirus nucleic acid or a functional part, derivative and/or analogue thereof. In one example said adenovirus nucleic acid comprises a host range  
20 mutation that enables adenovirus to replicate in non human primate cells.

In one aspect the invention provides a library obtainable by a method of the invention.

The invention further provides the use of a library  
25 obtainable by a method of the invention for determining at least one function of at least one nucleic acid present in a library of the invention.

The invention further provides in a method for  
30 amplifying a vehicle present in a library of the invention, comprising providing a cell with said vehicle, culturing said cell allowing the amplification of said vehicle and harvesting vehicles amplified by said cell. Preferably said cell is a primate cell. Thus enabling the amplification of  
35 vehicles comprising viral elements that allow replication of said vehicle nucleic acid. Preferably said cell comprises

nucleic acid encoding an adenovirus E1-region protein. Thus allowing among others the amplification of vehicles comprising viral elements derived from adenovirus comprising adenovirus nucleic acid comprising a functional deletion of at least part of the E1-region. Preferably said cell is a PER.C6 cell (ECACC deposit number 96022940) or a functional derivative and/or analogue thereof. A PER.C6 cell or a functional derivative and/or analogue thereof allows the replication of adenovirus nucleic acid with a deletion of the E1-coding region without concomitant production of replication competent adenovirus in instances wherein said adenovirus nucleic acid and chromosomal nucleic acid in said PER.C6 cell or functional derivative and/or analogue thereof do not comprise sequence overlap that allows homologous recombination between said adenovirus and chromosomal nucleic acid that leads to the formation of replication competent adenovirus.

Preferably, said cell further comprises nucleic acid encoding adenovirus E2A and/or an adenovirus E4-region protein or a functional part, derivative and/or analogue thereof. Thus allowing the replication of adenovirus nucleic acid with functional deletions of nucleic acid encoding adenovirus E2A and/or an adenovirus E4-region protein, thereby inhibiting replication of said adenovirus nucleic acid in a cell not comprising nucleic acid encoding adenovirus E2A and/or an adenovirus E4-region protein or a functional part, derivative and/or analogue thereof, for instance a cell capable of displaying a certain function.

In one example vehicle nucleic acid does not comprise sequence overlap with other nucleic acid present in said cell leading to the formation of vehicle nucleic acid capable of replicating in the absence of E1-region encoded proteins.

The invention further provides a library according to the invention or a method according to the invention, wherein said multiplicity of compartments comprises a multiwell

format. A multiwell format is very suited for automated execution of at least part of the methods of the invention.

In one aspect the invention provides a library  
5 wherein said at least one nucleic acid encodes a product of unknown function.

The library of the invention and/or the methods of the invention are preferably used or performed in an at least substantially automated setting.

10

The invention further provides a multiplicity of cells comprising a library according to the invention.

The present invention uses high-throughput generation of  
15 recombinant adenoviral vector libraries containing of one or more sample nucleic acids followed by high-throughput screening of the adenoviral vector libraries in a host to alter the phenotype of a host as a means of assigning a function to expression product(s) of the sample nucleic  
20 acids. Libraries of E1-deleted adenoviruses are generated in a high-throughput setting using nucleic acid constructs and transcomplementary packaging cells. The sample nucleic acid libraries can be a set of distinct defined or undefined sequences or can be a pool of undefined or defined sequences.  
25 The first nucleic acid construct is a relatively small and easy to manipulate adapter plasmid containing, in an operable configuration, at least a left ITR, a packaging signal, and an expression cassette with the sample nucleic acids. The second nucleic acid construct contains one or more nucleic  
30 acid molecules that partially overlap with each other and/or with sequences in the first construct and contains at least all adenovirus sequences necessary for replication and packaging of a recombinant adenovirus not provided by the adapter plasmid or packaging cells. The second nucleic acid  
35 construct is deleted in E1-region sequences and optionally E2B region sequences other than those required for virus generation and/or E2A, E3 and/or E4 region sequences.

Cotransfection of the first and second nucleic acid constructs into the packaging cells leads to homologous recombination between overlapping sequences in the first and second nucleic acid constructs and among the second nucleic acid constructs when it is made up of more than one nucleic acid molecule. Generally the overlapping sequences are no more than 5000 bp and encompass E2B region sequences essential for virus production. Recombinant viral DNA is generated with an E1-deletion that is able to replicate and propagate in the E1-complementing packaging cells to produce a recombinant adenovirus vector library. The adenovirus vector library is introduced into a host in a high-throughput setting which is grown to allow sufficient expression of the product(s) encoded by the sample nucleic acids to permit detection and analysis of its biological activity. The host can be cultured cells *in vitro* or an animal or plant model. Sufficient expression of the product(s) encoded by the sample nucleic acids alters the phenotype of the host. Using any of a variety of *in vitro* and or *in vivo* assays for biological activity, the altered phenotype is identified and analyzed and function is thereby assigned to the product(s) of the sample nucleic acids. The plasmid-based adenovirus vector systems described here provides for the creation of large gene-transfer libraries that can be used to screen for novel genes applicable to human diseases. Identification of a useful or beneficial biological effect of a particular adenovirus mediated transduction can result in a useful gene therapeutic product or a target for a small molecule drug for treatment of human diseases.

There are several advantages to the subject invention over currently available techniques. The entire process lends itself to automation especially when implemented in a 96-well or other multi-well format. The high-throughput screening using a number of different *in vitro* assays provides a means of efficiently obtaining function information in a relatively short period of time. The

member(s) of the recombinant adenoviral libraries that exhibit or induce a desired phenotype in a host *in vitro* or *in situ* are identified to collapse the libraries to a manageable number of recombinant adenovirus vectors or clones which can be tested *in vitro* in an animal model.

Another distinct advantage of the subject invention is that the methods produce RCA-free adenovirus libraries. RCA contamination throughout the libraries could become a major obstacle especially if libraries are continuously amplified for use in multiple screening programs. A further advantage of the subject invention is minimization of the number of steps involved in the process. The methods of the subject invention do not require cloning of each individual adenovirus before use in a high throughput screening program. Other systems include one or more steps in *E. coli* to achieve homologous recombination for the various adenoviral plasmids necessary for vector generation (Chartier et al., (1996) *J. Virol.* 70(7):4805-4810; Crouzet et al., (1997) *Proc. Natl. Acad. Sci.* 94(4):1414-1419; He et al., (1998) *Proc. Natl. Acad. Sci.* 95(5):2509-2514). Another plasmid system that has been used for adenoviral recombination and adenoviral vector generation and which is based on homologous recombination in human cells is the pBHG series of plasmids. However, this is used in 293 cells, the plasmids have overlap with E1 sequences plus the plasmid pBHG contains two ITRs closely together which leads to instability of the plasmid. All these features are undesirable and lead to RCA production or otherwise erroneous adenovirus vector production (Bett et al., (1994) *Proc. Natl. Acad. Sci. USA* 91(19):8802-8806). The recombinant nucleic acids of the subject invention have been designed to avoid constructions with these undesirable features.

A further advantage of the subject invention is the ability of recombinant adenoviruses to efficiently transfer and express recombinant genes in a variety of mammalian cells

and tissues *in vitro* and *in vivo* resulting in the high expression of the transferred sample nucleic acids. The ability to productively infect quiescent cells, further expands the utility of the recombinant adenovirus libraries.

5 In addition, high expression levels insure that the product(s) of the sample nucleic acids will be expressed to sufficient levels to induce a change in the phenotype of a host that can be detected and allow the function of the product(s) encoded by the sample nucleic to be determined.

10 The sample nucleic acids can be genomic DNA, cDNA, previously cloned DNA, genes, ESTs, synthetic double stranded oligonucleotides, or randomized sequences derived from one or multiple related or unrelated sequences and can be directly expressed as a polypeptide, antisense nucleic acid or genetic  
15 suppressor element (GSE). The sample nucleic acid sequences can be obtained from any organism including mammals (for example, man, monkey, mouse), fish (for example, zebrafish, pufferfish, salmon), nematodes (for example, *C. elegans*), insects (for example, *Drosophila*), yeasts, fungi, bacteria,  
20 and plants. Alternatively, the sample nucleic acids are prepared as synthetic oligonucleotides using commercially available DNA synthesizers and kits. The strand coding the open reading frame of the polypeptide or product of the sample nucleic acid and the complementary strand are prepared  
25 individually and annealed to form double-stranded DNA. Special annealing conditions are not required. The sequences of the sample nucleic acids can be randomized or not through mutagenizing or methodologies promoting recombination. The sample nucleic acids code for a product(s) for which a  
30 biological activity is unknown. The phrase biological activity is intended to mean an activity which is detectable or measurable either *in situ*, *in vivo* or *in vitro*. Examples of a biological activity include but are not limited to altered viability, morphologic changes, apoptosis induction,  
35 DNA synthesis, tumorigenesis, disease or drug susceptibility, chemical responsiveness or secretion, and protein expression.



The sample nucleic acids preferably contain compatible ends to facilitate ligation to the vector in the correct orientation and to operatively link the sample nucleic acids to a promoter. For the example of synthetic double-stranded oligonucleotide ligation, the ends compatible to the vector can be designed into the oligonucleotides. When the sample nucleic acid is ESTs, genomic DNA, cDNA, genes or a previously-cloned DNA, the compatible ends can be formed by restriction enzyme digestion or the ligation of linkers to the DNA containing the appropriate restriction enzyme sites. Alternatively, the vector can be modified by the use of linkers. The restriction enzyme sites are chosen so that transcription of the sample nucleic acid from the vector produces the desired product, i.e., polypeptide, antisense nucleic acid, or GSE.

The vector into which the sample nucleic acids are preferably introduced contains, in operable configuration, an ITR, at least one cloning site or preferably, a multiple cloning site, for insertion of a library of sample nucleic acids, and transcriptional regulatory elements for delivery and expression of the sample nucleic acid in a host. It generally does not contain E1 region sequences, E2B region sequences other than those required for late gene expression, E2A region sequences, E3 region sequences or E4 region sequences. The E1-deleted delivery vector or adapter plasmid is digested with the appropriate restriction enzymes, either simultaneously or sequentially, to produce the appropriate ends for directional cloning of the sample nucleic acid whether it be synthetic double-stranded oligonucleotides, genomic DNA, cDNA, ESTs, or a previously-cloned DNA. Restriction enzyme digestion is routinely performed using commercially available reagents according to the manufacturer's recommendations and varies according to the restriction enzymes chosen. A distinct set or pool of sample nucleic acids is inserted into E1-deleted adapter plasmid to produce a corresponding set or library of plasmids for the

production of adenovirus vectors. An example of an adapter plasmid is pMLPI.TK which is made up of adenovirus nucleotides 1-458, followed by the adenovirus major late promoter, functionally linked to the herpes simplex virus thymidine kinase gene, and followed by adenovirus nucleotides 3511-6095. Other examples of adapter plasmids are pAd/L420-HSA (Fig. 21) and pAd/Clip (Fig. 22). pAd/L420-HSA contains adenovirus nucleotides 1-454, the L420 promoter linked to the murine HSA gene, a poly-A signal followed by adenovirus nucleotides 3511-6095. pAd/CLIP was made from pAd/L420-HSA by replacement of the expression cassette (L420-HSA) with the CMV promoter, a multiple cloning site, an intron and a poly-A signal.

Once digested, the vector and sample nucleic acids are purified by gel electrophoresis. The nucleic acids can be extracted from various gel matrices by, for example, agarase digestion, electroelution, melting, or high salt extraction. In combination with these methods or alternatively, the digested nucleic acids can be purified by chromatography (e.g. Qiagen or equivalent DNA binding resins) or phenol-chloroform extraction and ethanol precipitation. The optimal purification method depends on the size and type of the vector and sample nucleic acids. Both can be used without purification. Generally, the sample nucleic acids contain 5'-phosphate groups and the vector is treated with alkaline phosphatase to promote nucleic acid-vector ligation and prevent vector-vector ligation. If the sample nucleic acid is a synthetic oligonucleotide, 5'-phosphate groups are added by chemical or enzymatic means. For ligation, molar ratios of sample nucleic acids (insert) to vector DNA range from approximately 10:1 to 0.1:1. The ligation reaction is performed using T4 DNA ligase or any other enzyme that catalyzes double-stranded DNA ligation. Reaction times and temperature can vary from about 5 minutes to 18 hours, to from about 15°C to room temperature, respectively.

The magnitude of expression can be modulated using promoters (CMV immediately early, promoter, SV40 promoter, retrovirus LTRs) that differ in their transcriptional activity. Operatively linking the sample nucleic acid to a strong promoter such as the CMV immediate early promoter and optionally one or more enhancer element results in higher expression allowing the use of a lower multiplicity of infection to alter the phenotype of a host. The option of using a lower multiplicity of infection increases the number of times a library can be used *in situ*, *in vitro* and *in vivo*. Moreover, the lower the virus library multiplicity of infection and dosages used in screening programs, assays and animal models decreases the chance of eliciting toxic effects in the transduced host, increasing again the reliability of the system subject of this invention. Another way to reduce possible toxic effects relating to expression of the library is to use a regulatable promoter, particularly one which is repressed during virus production, but which can be turned on or is active during functional screenings with the adenoviral library, to provide temporal and/or cell type specific control throughout the screening assay. In this way, sample nucleic acids that are members of the library and which are toxic or inhibitory to the complementing cell line or which in any other way interfere with adenovirus replication and production can still be produced as an adenoviral vector (see WO 97/20943). Examples of this type of promoter are the AP1-dependent promoters which are repressed by adenoviral E1 gene products, resulting in shut off of sample nucleic acid expression during adenoviral library production (see van Dam et al., (1990) *Mol. Cell. Biol.* 10(11):5857-5864). Such a promoter can be made using combinatorial techniques or natural or adapted forms of promoters can be included. Examples of AP1-dependent promoters are promoters from the collagenase, c-myc, monocyte chemoattractant protein (JE or mcp-1/JE) and stromelysin genes (Hagmeyer et al., (1993) *EMBO J.* 12(9);3559-3572; Offringa et al., (1990) *Cell* 62(23):527-

538; Offringa et al., (1988) *Nucleic Acids Res.* 16(23):10973-10984; van Dam et al., (1989) *Oncogene* 4(10):1207-1212).

Alternatively, other more specific but stronger promoters can be used especially when complex *in vitro* screenings are

5 employed or *in vivo* models are employed and tissue-regulated expression is desired. Any promoter/enhancer system

functional in the chosen host can be used. Examples of tissue-regulated promoters include promoters with specific activity or enhanced activity in liver, such as the albumin

10 promoter (Tronche et al., (1990) *Mol. Biol. Med.* 7(2):173-

185). Another approach to enhanced expression is to increase the half-life of the mRNA transcribed from the sample nucleic acids by including stabilizing sequences in the 3'

untranslated region. A second nucleic acid construct, a

15 helper plasmid having sequences homologous to sequences in the E1-deleted adapter plasmids, which carries all necessary adenoviral genes necessary for replication and packaging, also is prepared. Preferably, the helper plasmid has no

20 complementing sequences that are expressed by the packaging cells that would lead to production of replication competent adenovirus. In addition, preferably the helper plasmids,

adapter plasmid and packaging cell have no sequence overlap that would lead to homologous recombination and RCA

25 formation. The region of sequence overlap shared between the adapter plasmid and the helper plasmid allows homologous recombination and the formation of an E1-deleted,

replication-defective recombinant adenovirus genome.

Generally the region of overlap encompasses E2B region

30 sequences that are required for late gene expression. The amount of overlap which provides for the best efficiency without appreciably decreasing the size of the library insert can be determined empirically. The sequence overlap is generally 10 bp to 5000 bp, more preferably 2000 to 3000 bp.

35 The size of the sample nucleic acids or DNA inserts in a desired adenovirus library can vary from several hundred base pairs (e.g., sequences encoding neuropeptides) to more than

30 Kbp (e.g., titin). The cloning capacity of the adenoviral vectors produced using adapter plasmids can be increased by deletion of additional adenoviral gene(s) that are then easily complemented by a derivative of an E1-complementing cell line. As an example, candidate genes for deletion include E2, E3, and/or E4. For example, regions essential for adenovirus replication and packaging are deleted from the adapter and helper plasmids and expressed, for example, by the complementing cell line. For E3 deletions, genes in this region do not need to be complemented in the packaging cell for *in vitro* models, and for *in vivo* models, the impact upon immunogenicity of the recombinant virus can be assessed on an ad hoc basis.

The set or library of specific adapter plasmids or pool(s) of adapter plasmids is converted to a set or library of adenoviral vectors. The adapter plasmids containing the sample nucleic acids are linearized and transfected into an E1-complementing cell line preferably seeded in microtiter tissue culture plates with 96, 384, 1,536 or more wells per plate, together with helper plasmids having sequences homologous to sequences in the adapter plasmid and containing all adenoviral genes necessary for replication and packaging. Recombination between the homologous sequences shared by adapter and helper plasmids to generate an E1-deleted, replication-defective adenovirus genome that is replicated and packaged, preferably, in an E1-complementing cell line. If more than one helper plasmid is used, recombination between homologous regions shared among the helper plasmids on the one hand and homologous recombination with the adapter plasmid results in the formation of a replication-defective recombinant adenovirus genome. The regions of sequence overlap between among the adapter and helper plasmids can vary from about a few hundred nucleotides or greater. Recombination efficiency will increase by increasing the size of the overlap.

The E1-functions provided by the transcomplementing packaging cell permits the replication and packaging of the E1-deleted recombinant adenovirus genome. The adapter and/or helper plasmids preferably have no sequence overlap amongst themselves or with the complementing sequences in the packaging cells that can lead to the formation of replication competent adenovirus (RCA). Preferably, at least one of the ITRs on the adapter and helper plasmids is flanked by a restriction enzyme recognition site not present in the adenoviral sequences or expression cassette so that the ITR is freed from vector sequences by digestion of the DNA with that restriction enzyme. In this way, initiation of replication occurs more efficiently. In order to increase the efficiency of recombinant adenovirus generation higher throughput can be obtained by using microtiter tissue culture plates with 96, 384 or 1,536 wells per plate instead of using large tissue culture vials or flasks. E1-complementing cell lines are grown in microtiter plates and co-transfected with the libraries of adapter plasmids and a helper plasmid(s). Automation of the method using, for example, robotics can further increase the number of adenovirus vectors that can be produced (Hawkins et al., (1997) *Science* 276(5320):1887-9, Houston, (1997) *Methods Find. Exp. Clin. Pharmacol.* 19 Suppl. A:43-5).

As an alternative to the adapter plasmids, the sample nucleic acids can be ligated to "minimal" adenovirus vector plasmids. The so-called "minimal" adenovirus vectors according to the present invention retain at least a portion of the viral genome that is required for encapsidation of the genome into virus particles (the encapsidation signal), as well as at least one copy of at least a functional part or a derivative of the Inverted Terminal Repeat (ITR), that is DNA sequences derived from the termini of the linear adenovirus genome that are required for replication. The minimal vectors preferably are used for the generation and production of helper- and RCA-free stocks of recombinant adenovirus

vectors and can accommodate up to 38 kb of foreign DNA. The helper functions for the minimal adenovirus vectors are preferably provided in *trans* by encapsidation-defective, replication-competent DNA molecules that contain all the viral genes encoding the required gene products, with the exception of those genes that are present in the complementing cell, or genes that reside in the vector genome.

Packaging of the "minimal" adenovirus vector is achieved by cotransfection of an E1-complementing cell line with a helper virus or, alternatively, with a packaging deficient replicating helper system. Preferably, the packaging deficient replicating helper is amplified following transfection and expresses the sequences required for replication and packaging of the minimal adenovirus vectors that are not expressed by the packaging cell line. The packaging deficient replicating helper is not packaged into adenovirus particles because its size exceeds the capacity of the adenovirus virion and/or because it lacks a functional encapsidation signal. The packaging deficient replicating helper, the minimal adenovirus vector, and the complementing cell line, preferably, have no region of sequence overlap that permits RCA formation.

The replicating, packaging deficient helper molecule always contains all adenovirus coding sequences that produce proteins necessary for replication and packaging with or without the ones provided by the packaging cell line. Replication of the said helper molecule itself may or may not be mediated by adenovirus proteins and ITRs. Helper molecules that replicate through the activity of adenovirus proteins (that is E2-gene products acting together with cellular proteins) contain at least one ITR derived from adenovirus. The E2-gene products can be expressed by an E1-dependent or an E1-independent promoter. Where only one ITR is derived from an adenovirus, the helper molecule also

preferably contains a sequence that anneals in an intramolecular fashion to form a hairpin-like structure.

Following E2-gene product expression, the adenovirus DNA polymerase recognizes the ITR on the helper molecule and produces a single-stranded copy and the 3'-terminus intramolecularly anneals, forming a hairpin-like structure that serves as a primer for reverse strand synthesis. The product of reverse strand synthesis is a double-strand hairpin with an ITR at one end. This ITR is recognized by adenovirus DNA polymerase which produces a double-stranded molecule with an ITR at both termini (see e.g. Fig. 13) and becomes twice as long as the transfected molecule (in our example it duplicates from 35 Kb to 70 Kb). Duplication of the helper DNA enhances the production of sufficient levels of adenovirus proteins. Preferably, the size of the duplicated molecule exceeds the packaging capacity of the adenovirus virion and is, therefore, not packaged into adenovirus particles. The absence of a functional encapsidation signal in the helper molecule further insures that the helper molecule is packaging deficient. The produced adenoviral proteins mediate replication and packaging of the cotransfected or co-infected minimal vectors.

When the replication of the helper molecule is independent of adenovirus E2-proteins, the helper construct preferably contains an origin of replication derived from SV40. Transfection of this DNA together with the minimal adenoviral vector in an E1-containing packaging cell line that also inducibly expresses the SV40 Large T protein, or as much SV40 derived proteins as necessary for efficient replication, leads to replication of the helper construct and expression of adenoviral proteins. These then initiate replication and packaging of the co-transfected or co-infected minimal adenoviral vectors. There are preferably no regions of sequence overlap shared by the replication-deficient packaging constructs, the minimal adenovirus



vectors, and the complementing cell lines that may lead to the generation of RCA.

It is to be understood that during propagation of the minimal adenoviral vectors each amplification step on E1-complementing cells is preceded by transfection of any of the described helper molecules since minimal vectors by themselves can not replicate on E1-complementing cells. Alternatively, a cell line that contains all the adenoviral genes necessary for replication and packaging stably integrated in the genome and that can be excised and replicated conditionally can be used. (Valerio and Einerhand PCT/NL9800061).

Transfection of nucleic acid into cells is required for packaging of recombinant vectors into virus particles and can be mediated by a variety of chemicals including liposomes, DEAE-dextran, polybrene, and phosphazenes or phosphazene derivatives (WO97/07226). Liposomes are available from a variety of commercial suppliers and include DOTAP® (Boehringer-Mannheim), Tfx®-50, Transfectam®, ProFection® (Promega, Madison, WI), and LipofectAmin®, Lipofectin®, LipofectAce® (GibcoBRL, Gaithersburg, MD). In solution, the lipids form vesicles that associate with the nucleic acid and facilitate its transfer into cells by fusion of the vesicles with cell membranes or by endocytosis. Other transfection methods include, electroporation, calcium phosphate coprecipitation, and microinjection. If transfection conditions for a given cell line have not been established or are unknown, they can be determined empirically (Maniatis et al., Molecular Cloning, pages 16.30-16.55).

The yield of recombinant adenovirus virus vectors can be increased by denaturing the double stranded plasmid DNAs before transfection into an E1 complementing cell line. Denaturing can be by melting double-stranded DNAs at, for example, 95-100°C, followed by rapid cooling using various ratios of the adapter and helper plasmids that have overlapping sequences. Also a PER.C6 derivative that stably

or transiently expresses E2A (DNA binding protein) and/or E2B gene (pTP-Pol) could be used to increase the adenovirus production per well by increasing the replication rate per cell. Alternatively, cotransfection of recombinase protein(s) or recombinase DNA expression construct(s), i.e. recombinase from *Kluyveromyces waltii*, (Ringrose et al., (1997) *Eur. J. Biochem.* 248(3):903-912), or any other gene or genes encoding factors that can increase homologous recombination efficiency can be used. The inclusion of 0.1-100 mM sodium butyrate during transfection and/or replication on the packaging cells can increase virus production. These improvements will result in improved virus yields per microtiter well and thus the number and type of tests that can be done with one single library will increase and may overcome variability between the various genes or sample nucleic acids in a library.

The cell lines used for the production of adenovirus vectors that do express E1 region products includes, for example, 293 cells, PER.C6 (ECACC 96022940), or 911 cells. Each of these cell lines have been transfected with nucleic acids that encode for the adenovirus E1 region. These cells stably express E1 region gene products and have been shown to package E1-deleted recombinant adenovirus vectors. Yields of recombinant adenovirus obtained on PER.C6 cells are higher than obtained on 293 cells.

Each of these cell lines provide the basis for introduction of e.g. E2B or E2A constructs (e.g. ts125E2A and/or hrE2A), or E4 etc., that permit the propagation of adenovirus vectors that have mutations, deletions or insertions in the corresponding genes. These cells can be modified to express additional adenovirus gene products by the introduction of recombinant nucleic acids that stably express the appropriate adenovirus genes or recombinant nucleic acids can be introduced that transiently express the appropriate gene(s), for example, the packaging deficient replicating helper molecules or the helper plasmids.

All or nearly all trans complementing cells grown in microtiter plate wells (96, 384, 1,536 or more wells) produce recombinant adenovirus following transfection with either the adapter plasmid or the minimal adenovirus plasmid library and the appropriate helper molecule(s). A large number of adenovirus gene transfer vectors or a library, each expressing a unique gene, can thus be conveniently produced on a scale that allows analysis of the biological activity of the particular gene products both *in vitro* and *in vivo*. Due to the wide tissue tropism of adenoviral vectors, a large number of cell and tissue types are transducible with an adenoviral library.

Libraries of genes or sample nucleic acids preferably are converted using the above methods to RCA free adenoviral libraries. The adenoviral libraries of genes or sample nucleic acids with unknown function are then used to perform high-throughput screening involving a number of *in vitro* assays, such as immunological assays including ELISAs, proliferation assays, drug resistance assays, enzyme activity assays, organ cultures, differentiation assays and cytotoxicity assays. Adenoviral libraries can be tested on tissues or tissue sections or tissue derived primary short-lived cell cultures including primary endothelial and smooth muscle cell cultures (Wijnberg et al., (1997) *Thromb Haemost* 78(2), 880-6), coronary artery bypass graft libraries, (Vassalli et al., (1997) *Cardiovasc Res.* 35(3), 459-69; Fuster and Chesebro, (1985) *Adv. Prostaglandin Thromboxane Leukot Res.* 13, 285-99), umbilical cord tissue including HUVEC (Gimbrone, (1976) *Prog. Hemost. Thromb.* 3, 1-28; Striker et al., (1980) *Methods Cell. Biol.* 21A, 135-51), couplet hepatocytes (Graf et al., (1984) *Proc. Natl. Acad. Sci. USA* 81(20), 6516-20), and epidermal cultures (Fabre, (1991) *Immunol. Lett.* 29(1-2), 161-5; Phillips, (1991) *Transplantation* 51(5), 937-41). Plant cell cultures, including suspension cultures, can also be used as host cells

for the adenoviral libraries carrying any DNA sequence, including human derived DNA sequences and plant derived sequences. (de Vries et al., (1994) *Biochem. Soc. Symp.* 60, 43-50; Fukada et al., (1994) *Int. J. Devel. Biol.* 38(2), 287-99; Jones, (1983) *Biochem. Soc. Symp.* 48, 221-32; Kieran et al., (1997) *J. Biotechnol.* 59(1-2), 39-52; Stanley, (1993) *Curr. Opin. Genet. Dev.* 3(1), 91-6; Taticek et al., (1994) *Curr. Opin. Biotechnol.* 5(2), 165-74.

Depending on the size of the initial unselected library, once an adenoviral library of genes has been collapsed by *in vitro* assays to a reasonable number of candidates, the adenoviruses can be tested in appropriate animal models. Examples of animal models that can be used include models for Alzheimer's disease, arteriosclerosis, transgenic animals which have altered expression of endogenous or exogenous genes including mice with gene(s) that have been inactivated, animals with cancers implanted at specific sites, cancer metastasis models, Parkinson disease models, human bone marrow chimeric mice such as NOD-SCID mice, and the like. As additional testing is required, the stocks of candidate adenoviruses can be expanded by passaging the adenoviruses under the appropriate transcomplementing conditions.

Depending on the animal model used adenoviral vectors or mixtures of pre-selected pools of adenoviral vectors can be instilled or applied or administered at appropriate sites in the desired animal such as lung (Sene et al., (1995) *Hum. Gene Ther.* 6(12):1587-93) in non-human primates, brain of normal and apoE deficient mice (Robertson et al., (1998) *Neuroscience* 82(1):171-80.) for Alzheimer disease (Walker et al., (1997) *Brain Res. Brain Res. Rev.* 25(1):70-84) and Parkinson disease models (Hockman et al., (1971) *Brain Res.* 35(2):613-8.; Zigmond and Stricker, (1984) *Life Sci.* 35(1):5-18.), injected in the blood stream (e.g. intravenous) for liver disease models including liver failure and Wilson

disease (Cuthbert, (1995) *J. Investig. Med.* 43(4):323-36; Karrer et al., (1984) *Curr. Surg.* 41(6):464-7) and tumor models including metastases models (Esandi et al., (1997) *Gene Ther.* 4(4):280-7; Vincent et al., (1996) *J. Neurosug.* 85(4):648-54; Vincent et al., (1996) *Hum. Gene Ther.*

7(2):197-205). Injection of selected adenoviral vectors directly into the bone marrow of human chimeric NOD-SCID mice (Dick et al., (1997) *Stem Cells* 15 Suppl. 1:199-203; Mosier et al., (1988) *Nature* 335(6187):256-9). Finally selected  
10 adenovirus can be applied locally in for example the disease vascular tissue of restenosis animal models (Karas et al., (1992) *J. Am. Coll. Cardiol.* 20(2):467-74).

In addition, wet laboratory assays can be complemented by using an electronic version of the sequence database on  
15 which the adenoviral library is built. This allows, for example, protein motif searching and thus linking of new members of a family to known members with known function of the same family. The use of 'Hidden Markow Models' (HMMs) (Eddy. (1996) *Proc. Natl. Acad. Sci. USA* 94(4):1414-1419)

20 allows the establishment of novel families by distilling out essential features of a family and building a model of what the members should look like. Finally, this can be combined with structural data by using the threading approach using a known structure as the thread and trying to find putative  
25 structure without having determined the actual structure of the novel protein (Rastan and Beeley (1997) *Curr. Opin. Genet. Dev.* 7 (6):777-83).

Naturally, the functional data obtained using adenoviral libraries made in accordance with the methods disclosed in this application is the foundation  
30 of the endeavor to find novel genes with expected or desired functions and will be the core of functional genomics.

Finally, once the number of adenovirus vectors is at a level at which animal experiments can be performed, another addition to the method is to grow up the selection of  
35 candidate adenovirus vectors carrying the candidate genes.

This can then be followed by purification of the clones by, for example, using adenovirus tagged in the Hi loop of the knob domain of the fiber. Alternatively, large scale HPLC analysis can be used in a semipreparative fashion to yield partially purified adenovirus samples for animal experiments or *in vitro* screenings where more purified adenovirus preparations are desired. Therefore, the described method and reagents allow rapid transfer of a collection of genes to *in vivo* studies of a limited number of animals which otherwise would be unfeasible. The automation of each of the steps of the procedure using robotics will further enhance the number of genes and sample nucleic acids that can be functionated.

15 In one aspect the invention provides a method of producing a recombinant adenovirus vector library, said method comprising:

growing a cell culture containing a plurality of cells comprising adenovirus E1-complementing sequences with

20 i) an adapter plasmid library comprising an adapter plasmid based on or derived from an adenovirus having no E1 region sequences which overlap with E1 region sequences in said plurality of cells or a recombinant nucleic acid to be inserted into said packaging cell and would lead to generation of replication competent adenovirus in said plurality of cells, and no E2B region sequences other than essential E2B sequences, no E2A region sequences, no E3 region sequences and no E4 region sequences and having in operable configuration a functional Inverted Terminal Repeat, a functional encapsidation signal, and sufficient adenoviral sequences which allow for homologous recombination with said recombinant nucleic acid, and a library of sample nucleic acids inserted into said adapter plasmid operatively linked to a promoter; and

ii) a recombinant nucleic acid based on or derived from an adenovirus, wherein said recombinant nucleic acid comprises in operable configuration a functional Inverted Terminal Repeat and sufficient adenovirus sequences for replication, wherein said recombinant nucleic acid partially overlaps with said adapter plasmid library which allow for homologous recombination leading to replication-defective, recombinant adenovirus;

under conditions whereby a recombinant adenovirus vector library is produced.

Preferably, at least one of said adapter plasmid library and said recombinant nucleic acid are heat denatured prior to transfecting said plurality of cells or ancestors of said plurality of cells.

Preferably, said adenovirus E1-complementing sequences, said adapter plasmid library and said recombinant nucleic acid have no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which they are transferred.

In another aspect the invention provides a method of producing a recombinant adenovirus vector library, said method comprising:

growing a cell culture containing a plurality of cells comprising adenovirus E1 complementing sequences with

i) a recombinant nucleic acid library comprising a first recombinant nucleic acid based on or derived from an adenovirus, comprising in operable configuration two functional Inverted Terminal Repeats, one functional encapsidation signal, and having no functional adenovirus genes and a library of sample nucleic acids inserted into said first recombinant nucleic acid operatively linked to a promoter; and

ii) a second recombinant nucleic acid based on or derived from an adenovirus comprising in operable configuration two functional Inverted Terminal Repeats, and sufficient adenovirus sequences for replication, wherein said  
5 second recombinant nucleic acid comprises a deletion of at least the E1 region and encapsidation signal of said adenovirus;

under conditions whereby a recombinant adenovirus vector library is produced.

10 Preferably said cell culture is in a multiwell format.

Preferably, said adenovirus E1-complementing sequences, said first recombinant nucleic acid and said second recombinant nucleic acid have no overlapping sequences which allow for homologous recombination leading to replication  
15 competent virus in a cell into which they are transferred.

Preferably, said cell culture is a PER.C6 cell culture.

In one example, growth medium of said cell culture contains sodium butyrate in an amount sufficient to enhance production of said recombinant adenovirus vector library.

20 Preferably, said plurality of cells further comprises at least one of an adenovirus preterminal protein and a polymerase complementing sequence.

Preferably, said plurality of cells further comprises an adenovirus E2 complementing sequence. Preferably, said E2  
25 complementing sequence is an E2A complementing sequence or an E2B complementing sequence.

In one aspect said plurality of cells further comprises a recombinase protein, whereby said homologous recombination  
30 leading to replication-defective, recombinant adenovirus is enhanced. Preferably, said recombinase protein is a *Kluyveromyces waltii* recombinase.



In another aspect said plurality of cells further comprises a nucleotide sequence coding for a recombinase protein. Preferably, said recombinase protein is *Kluyveromyces waltii* recombinase.

5

In one aspect the members of said recombinant adenovirus vector library are identical.

In one aspect said promoter is an inducible promoter. Preferably, said promoter is repressed or down modulated by an adenovirus E1 gene product. In one aspect said promoter comprises an AP1 dependent promoter. Preferably, said AP1 dependent promoter is derived from a collagenase, a c-myc, a monocyte chemoattractant protein or a stromelysin gene.

15 In one aspect said sample nucleic acids encode a product of unknown function.

In another aspect said sample nucleic acids are selected from the group consisting of synthetic oligonucleotides, DNAs, cDNAs, genes, ESTs, antisense nucleic acids, or genetic suppressor elements.

20 In one aspect the invention provides a method for assigning a function to products encoded by sample nucleic acids, said method comprising:

25 growing a host cell containing a recombinant adenovirus vector library produced according to the method of the invention, whereby products encoded by said sample nucleic acids are expressed to produce at least one altered phenotype in said host cell; and

30 identifying said at least one altered phenotype, whereby a function is assigned to said products encoded by said sample nucleic acids.

Preferably, said host cell is a plant cell or an animal cell. Preferably, said animal cell is a human cell.

In one aspect said host cell is a member of a cell culture. Preferably, said cell culture is in a multiwell format.

5            Preferably a method of the invention is automated.

The invention further provides a non-human host cell containing a recombinant replication-defective adenovirus vector library.

10           The invention further provides a non-human host cell containing a recombinant replication-defective adenovirus vector library, wherein said replication-defective adenovirus vector library is produced by the method according to a method of the invention.

15           The invention further provides an isolated host cell containing a replication-defective adenovirus vector library.

The invention further provides an isolated host cell  
20           containing a replication-defective adenovirus vector library, wherein said replication-defective adenovirus vector library is produced by the method according to the invention. Preferably, said host cell is a human cell.

25           The invention further provides a method of producing a recombinant adenovirus vector library, said method comprising:

             growing a cell culture containing a plurality of cells expressing adenovirus E1-region sequences and expressing one  
30           or more functional gene products encoded by at least one adenovirus region selected from an E2A region and an E4 region with

             i) an adapter plasmid library comprising an adapter plasmid based on or derived from an adenovirus having no E1

region sequences which overlap with E1 region sequences in said plurality of cells or a recombinant nucleic acid to be inserted into said packaging cell , and no E2B region sequences other than essential E2B sequences, no E2A region sequences, no E3 region sequences and no E4 region sequences and having in operable configuration a functional Inverted Terminal Repeat, a functional encapsidation signal, and sufficient adenoviral sequences which allow for homologous recombination with said recombinant nucleic acid, and a library of sample nucleic acids inserted into said adapter plasmid operatively linked to a promoter; and

ii) a recombinant nucleic acid based on or derived from an adenovirus having no E1 region sequences which overlap with E1 sequences in said plurality of cells, and having no E2A region sequences or E4 region sequences expressed in said plurality of cells which would lead to production of replication competent adenovirus and having in operable configuration a functional adenovirus Inverted Terminal Repeat and sufficient adenovirus sequences for replication in said plurality of cells, wherein said recombinant nucleic acid has sufficient overlap with said adapter plasmid to provide for homologous recombination leading to production of recombinant adenovirus in said packaging cell.;

under conditions whereby a recombinant adenovirus vector library is produced in said plurality of cells.

Preferably, said recombinant nucleic acid further has no E3 region sequences.

Preferably, said plurality of cells expresses at least one functional E2A gene product.

Preferably, said at least one functional E2A gene product is a mutated gene product. Preferably, said mutated gene product is temperature sensitive.

Preferably, at least one of said adapter plasmid library and said recombinant nucleic acid are heat denatured prior to transfecting said plurality of cells or ancestors of said plurality of cells.

5

Preferably, said plurality of cells expresses one or more functional gene product encoded by E2B region sequences and wherein E2B region sequences for said functional E2B region gene products, other than those required for virus generation, are deleted from said recombinant nucleic acid, and optionally up to all E2B gene region sequences are deleted from said adapter plasmid.

Preferably, said plurality of cells expresses all gene products encoded by E2B region sequences, and wherein E2B region sequences for said functional E2B region gene products, other than those required for virus generation, are deleted from said recombinant nucleic acid, and optionally up to all E2B gene region sequences are deleted from said adapter plasmid.

Preferably, said cell culture is a PER.C6 cell culture.  
Preferably, said promoter is an inducible promoter.

In one aspect the invention provides a plurality of cells containing a recombinant replication-defective adenovirus vector library, wherein said recombinant replication-defective adenovirus vector library is produced according to a method of the invention. Preferably, said plurality of cells are PER.C6 cells.

The invention further provides a recombinant nucleic acid comprising:

a nucleic acid based on or derived from an adenovirus having no E1 region sequences which would lead to production of replication competent adenovirus in a packaging cell into which it is introduced and having in operable configuration a functional adenovirus Inverted Terminal Repeat and sufficient adenovirus sequences for replication in said packaging cell, wherein said nucleic acid has sufficient overlap with an adapter plasmid to provide for homologous recombination leading to production of recombinant adenovirus in said packaging cell. Preferably, said recombinant nucleic acid has at least one of no E2A region sequences or no E4 region sequences which are expressed in said packaging cell and would lead to production of recombinant adenovirus in said packaging cell. Preferably, said recombinant nucleic acid has no E2B region sequences, other than essential E2B region sequences for virus generation, which are expressed in said packaging cell. Preferably, said recombinant nucleic acid has no E3 region sequences. Preferably, said sufficient overlap is about 10 bp to about 5000 bp. Preferably, said sufficient overlap is about 2000 bp to about 3000 bp. Preferably, said sufficient overlap comprises E2B region sequences essential for virus generation.

The invention further provides an adapter plasmid comprising:

a nucleic acid based on or derived from an adenovirus having no E1 region sequences which overlap with E1 region sequences in a packaging cell into which it is introduced and would lead to production of replication competent adenovirus and no E2B region sequences other than essential E2B sequences, no E2A region sequences, no E3 region sequences and no E4 region sequences which overlap with other nucleic acid to be inserted into said packaging cell or contained in said packaging cell, and having in operable configuration a

functional Inverted Terminal Repeat, a functional encapsidation signal, and sufficient adenoviral sequences which allow for homologous recombination with said other nucleic acid leading to replication-defective, recombinant adenovirus, and a cloning site or a multiple cloning site. Preferably, said cloning site or said multiple cloning site is operably linked to a promoter. Preferably, said promoter is an inducible promoter. Preferably, said promoter is repressed or down modulated by an adenovirus E1 gene product. Preferably, said promoter comprises an AP1 dependent promoter. Preferably, said AP1 dependent promoter is derived from a collagenase gene, a c-myc gene, a monocyte chemoattractant protein gene or a stromelysin gene. Preferably, a library of sample nucleic acids is inserted into said multiple cloning site.

Preferably, a method of the invention is automated.

## EXAMPLES

### Example 1

#### Generation of cell lines able to transcomplement E1 defective recombinant adenovirus vectors

##### 911 cell line

A cell line that harbors E1 sequences of adenovirus type 5, able to trans-complement E1 deleted recombinant adenovirus has been generated (Fallaux et al, (1996) *Hum. Gene Ther.* 7: 215-222). This cell line was obtained by transfection of human diploid human embryonic retinoblasts (HER) with pAd5XhoIC, that contains nt. 80-5788 of Ad 5; one of the resulting transformants was designated 911. This cell line has been shown to be useful in the propagation of E1 defective recombinant adenovirus. It was found to be superior to the 293 cells. Unlike 293 cells, 911 cells lack

a fully transformed phenotype, which most likely is the cause of performing better as adenovirus packaging line:

plaque assays can be performed faster (4-5 days instead of 8-14 days on 293)

5 monolayers of 911 cells survive better under agar overlay as required for plaque assays

higher amplification of E1-deleted vectors.

10 In addition, unlike 293 cells that were transfected with sheared adenoviral DNA, 911 cells were transfected using a defined construct. Transfection efficiencies of 911 cells are comparable to those of 293.

#### New packaging constructs

##### Source of adenovirus sequences

15 Adenovirus sequences are derived either from pAd5.SalB, containing nt. 80-9460 of human adenovirus type 5 (Bernards et al, (1983) *Virology* 127:45-53) or from wild-type Ad5 DNA. PAd5.SalB was digested with *SalI* and *XhoI* and the large fragment was religated and this new clone was named pAd5.X/S.  
20 The pTN construct (constructed by Dr. R. Vogels, IntroGene, The Netherlands) was used as a source for the human PGK promoter and the NEO gene.

##### Human PGK promoter and NEO<sup>R</sup> gene

25 Transcription of E1A sequences in the new packaging constructs is driven by the human PGK promoter (Michelson et al, (1983) *Proc. Natl. Acad. Sci. USA* 80:472-476); Singer-Sam et al, (1984) *Gene* 32: 409-417), derived from plasmid pTN (gift of R. Vogels), which uses pUC119 (Vieira et al, (1987)  
30 pp. 3-11: *Methods in Enzymology*, Acad. Press Inc.) as a backbone. This plasmid was also used as a source for the NEO gene fused to the Hepatitis B Virus (HBV) poly-adenylation signal.

35 Fusion of PGK promoter to E1 genes (Fig. 1)

In order to replace the E1 sequences of Ad5 (ITR, origin of replication and packaging signal) by heterologous sequences we have amplified E1 sequences (nt.459 to nt.960) of Ad5 by PCR, using primers Ea1 (SEQ ID NO:27) and Ea2 (SEQ ID NO:28) (see Table I). The resulting PCR product was  
5 digested with *Cla*I and ligated into Bluescript (Stratagene), predigested with *Cla*I and *Eco*RV, resulting in construct pBS.PCRI.

Vector pTN was digested with restriction enzymes *Eco*RI  
10 (partially) and *Sca*I, and the DNA fragment containing the PGK promoter sequences was ligated into pBS.PCRI digested with *Sca*I and *Eco*RI. The resulting construct pBS.PGK.PCRI contains the human PGK promoter operatively linked to Ad5 E1 sequences from nt.459 to nt.916.

15

#### Construction of pIG.E1A.E1B (Fig. 2)

FIG.E1A.E1B.X contains the E1A and E1B coding sequences under the direction of the PGK promoter. As Ad5 sequences from nt.459 to nt.5788 are present in this construct, also  
20 pIX protein of adenovirus is encoded by this plasmid. pIG.E1A.E1B.X was made by replacing the *Sca*I-*Bsp*EI fragment of pAT-X/S by the corresponding fragment from pBS.PGK.PCRI (containing the PGK promoter linked to E1A sequences).

#### Construction of pIG.E1A.NEO (Fig. 3)

In order to introduce the complete E1B promoter and to fuse this promoter in such a way that the AUG codon of E1B 21 kD exactly functions as the AUG codon of NEO<sup>R</sup>, the E1B promoter was amplified using primers Ea3 (SEQ ID NO:29) and  
30 Ep2 (SEQ ID NO:30), where primer Ep2 introduces a *Nco*I site in the PCR fragment. The resulting PCR fragment, named PCRII, was digested with *Hpa*I and *Nco*I and ligated into pAT-X/S, which was predigested with *Hpa*I and with *Nco*I. The resulting plasmid was designated pAT-X/S-PCR2. The *Nco*I-*Stu*I  
35 fragment of pTN, containing the NEO gene and part of the



Hepatitis B Virus (HBV) poly-adenylation signal, was cloned into pAT-X/S-PCR2 which had been digested with *NcoI* and *NruI*). The resulting construct was pAT-PCR2-NEO. The poly-adenylation signal was completed by replacing the *ScaI-SalI* fragment of pAT-PCR2.NEO with the corresponding fragment of pTN, resulting in pAT.PCR2.NEO.p (A). The *ScaI-XbaI* of pAT.PCR2.NEO.p (A) was replaced with the corresponding fragment of pIG.E1A.E1B-X, containing the PGK promoter linked to E1A genes. The resulting construct was named pIG.E1A.NEO, and thus contains Ad5 E1 sequences (nt.459 to nt.1713) under the control of the human PGK promoter.

#### Construction of pIG.E1A.E1B (Fig. 4)

pIG.E1A.E1B contains nt.459 to nt.3510 of Ad5, that encode the E1A and E1B proteins. The E1B sequences are terminated at the splice acceptor at nt.3511. No pIX sequences are present in this construct.

pIG.E1A.E1B was made as follows: The sequences encoding the N-terminal amino acids of E1B 55kd were amplified using primers Eb1 (SEQ ID NO:31) and Eb2 (SEQ ID NO:32) which introduces a *XhoI* site. The resulting PCR fragment was digested with *BglIII* and cloned into *B1III/NruI* of pAT-X/S, thereby obtaining pAT-PCR3. The HBV poly (A) sequences of pIG.E1A.NEO were introduced downstream of the E1B sequences of pAT-PCR3 by exchange of the *Xba-SalI* fragment of pIG.E1A.NEO and the *XbaI XhoI* fragment of pAT.PCR3.

#### Construction of pIG.NEO (Fig. 5)

This construct is of use when established cells are transfected with E1A.E1B constructs and NEO selection is required. Because NEO expression is directed by the E1B promoter, NEO resistant cells are expected to co-express. E1A, which also is advantageous for maintaining high levels of expression of E1A during long-term culture of the cells. pIG.NEO was generated by cloning the *HpaI-ScaI* fragment of

pIG.E1A.NEO, containing the NEO gene under the control of the Ad5 E1B promoter, into pBS digested with *EcoRV* and *ScaI*.

#### Testing of constructs

5       The integrity of the constructs pIG.E1A.NEO, pIG.E1A.E1B.X and pIG.E1A.E1B was assessed by restriction enzyme mapping; furthermore, parts of the constructs that were obtained by PCR analysis were confirmed by sequence analysis. No changes in the nucleotide sequence were found.

10       The constructs were transfected into primary BRK (Baby Rat Kidney) cells and tested for their ability to immortalize (pIG.E1A.NEO) or fully transform (pAd5.XhoIC, pIG.E1A.E1B.X and pIG.E1A.E1B) these cells. Kidneys of 6-day old WAG-Rij rats were isolated, homogenized and trypsinized.

15       Subconfluent dishes (diameter 5 cm) of the BRK cell cultures were transfected with 1 or 5 µg of pIG.NEO, pIG.E1A.NEO, pIG.E1A.E1B, pIG/E1A.E1B.X, pAd5XhiIC, or with pIG.E1A.NEO together with PDC26 (Elsen *et al*, (1983) *Virology* 128:377-390), carrying the Ad5.E1B gene under control of the SV40

20       early promoter. Three weeks post-transfection, when foci were visible, the dishes were fixed, Giemsa stained and the foci counted.

      An overview of the generated adenovirus packaging constructs, and their ability to transform BRK, is presented

25       in Fig. 6. The results indicate that the constructs pIG.E1A.E1B and pIG.E1A.E1B.X are able to transform BRK cells in a dose-dependent manner. The efficiency of transformation is similar for both constructs and is comparable to what was found with the construct that was used to make 911 cells,

30       namely pAd5.XhoIC.

      As expected, pIG.E1A.NEO was hardly able to immortalize BRK. However, co-transfection of an E1B expression construct (PDC26) did result in a significant increase of the number of transformants (18 versus 1), indicating that the E1A encoded

35       by pIG.E1A.NEO is functional. We conclude therefore, that

the newly generated packaging constructs are suitable for the generation of new adenovirus packaging lines.

Generation of cell lines with new packaging constructs cell lines and cell culture

Human A549 bronchial carcinoma cells (Shapiro et al, (1978) *Biochem. Biophys. Acta* 530:197-207), human embryonic retinoblasts (HER), Ad5-E1-transformed human embryonic kidney (HEK) cells (293; Graham et al, (1977) *J. Gen. Virol.* 36: 59-72), and Ad5-transformed HER cells (911; Fallaux et al, (1996). *Hum. Gene Ther.* 7: 215-222) and PER cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and antibiotics in a 5% CO<sub>2</sub> atmosphere at 37°C. Cell culture media, reagents and sera were purchased from Gibco Laboratories (Grand Island, NY). Culture plastics were purchased from Greiner (N,rtingen, Germany) and Corning (Corning, NY).

Viruses and virus techniques

The construction of recombinant adenoviral vectors IG.Ad.MLP.nls.lacZ, IG.Ad.MLP.luc, IG.Ad.MLP.TK and IG.Ad.CMV.TK is described in detail in patent application EP 95202213. The recombinant adenoviral vector IG.Ad.MLP.nls.lacZ contains the *E. coli* lacZ gene, encoding  $\beta$ -galactosidase, under control of the Ad2 major late promoter (MLP), IG.Ad.MLP.luc contains the firefly luciferase gene drive by the Ad2 MLP, and adenoviral vectors IG.Ad.MLP.TK and IG.Ad.CMV.TK contain the Herpes Simplex Virus thymidine kinase (TK) gene under the control of the Ad2 MLP and the Cytomegalovirus (CMV) enhancer/promoter, respectively.

Transfections

All transfections were performed by calcium-phosphate precipitation DNA (Graham et al, (1973) *Virology* 52: 456-467) with the GIBCO Calcium Phosphate Transfection System (GIBCO

BRL Life Technologies, Inc., Gaithersburg, USA), according to the manufacturer's protocol.

#### Western blotting

5 Subconfluent cultures of exponentially growing 293, 911 and Ad5-E1-transformed A549 and PER cells were washed with PBS and scraped in Fos-RIPA buffer (10 mM Tris (pH 7,5), 150 mM NaCl, 1% NP40, 0,01% sodium dodecyl sulfate (SDS), 1% NA-DOC, 0,5 mM phenyl methyl sulfonyl fluoride (PMSF), 0,5 mM trypsin  
10 inhibitor, 50 mM NaF and 1 mM sodium vanadate). After 10 min. at room temperature, lysates were cleared by centrifugation. Protein concentrations were measured with the BioRad protein assay kit, and 25 µg total cellular protein was loaded on a 12.5% SDS-PAA gel. After  
15 electrophoresis, proteins were transferred to nitrocellulose (1h at 300 mA). Prestained standards (Sigma, USA) were run in parallel. Filters were blocked with 1% bovine serum albumin (BSA) in TBST (10 mM Tris, pH 8, 15 mM NaCl, and 0.05% Tween-20) for 1 hour. First antibodies were the mouse  
20 monoclonal anti-Ad5-E1B-55-kDa antibody A1C6 (Zantema et al, unpublished), the rat monoclonal anti-Ad5-E1B-221-kDa antibody C1G11 (Zantema et al, (1985) *Virology* 142:44-58). The second antibody was a horseradish peroxidase-labeled goat anti-mouse antibody (Promega). Signals were visualized by  
25 enhanced chemoluminescence (Amersham Corp. UK).

#### Southern blot analysis

High molecular weight DNA was isolated and 10 µg was digested to completion and fractionated on a 0.7% agarose  
30 gel. Southern blot transfer to Hybond N<sup>+</sup> (Amersham, UK) was performed with a 0.4 M NaOH, 0.6 M NaCl transfer solution (Church and Gilbert, 1984). Hybridization was performed with a 2463-nt *SspI-HindIII* fragment from pAd5.SalB (Bernards et al, (1983) *Virology* 127:45-53). This fragment consists of  
35 Ad5 bp. 342-2805. The fragment was radiolabeled with α-<sup>32</sup>P=dCTP with the use of random hexanucleotide primers and

Kelnow DNA polymerase. The southern blots were exposed to a Kodak XAR-5 film at -80°C and to a Phospho-Imager screen which was analyzed by B&L systems Molecular Dynamics Software.

5

#### A549

Ad5-E1-transformed A549 human bronchial carcinoma cell lines were generated by transfection with pIG.E1A.NEO and selection for G418 resistance. Thirty-one G418 resistant clones were established. Co-transfection of pIG.E1A.E1B with pIG.NEO yielded seven G418 resistant cell lines.

#### PER

Ad5-E1-transformed human embryonic retina (HER) cells were generated by transfection of primary HER cells with plasmid pIG.E1A.E1B. Transformed cell lines were established from well-separated foci. We were able to establish seven clonal cell lines, which we called PER.C1, PER.C3, PER.C4, PER.C5, PER.C6, PER.C8 and PER.C9. One of the PER clones, namely PER.C6, has been deposited at the ECACC under number 96022940.

#### Expression of Ad5 E1A and E1B genes in transformed A549 and PER cells

Expression of the Ad5 E1A and the 55-kDa and 21 kDa E1B proteins in the established A549 and PER cells was studied by means of Western blotting, with the use of monoclonal antibodies (mAb). mAb M73 recognizes the E1A products, whereas Mabs AIC6 and ClG11 are directed against the 55-kDa and 21 kDa E1B proteins, respectively. The antibodies did not recognize proteins in extracts from the parental A549 or the primary HER cells (data not shown). None of the A549 clones that were generated by co-transfection of pIG.NEO and pIG.E1A.E1B expressed detectable levels of E1A or E1B proteins (not shown). Some of the A549 clones that were generated by transfection with pIG.E1A.NEO expressed the Ad5

E1A proteins (Fig. 7), but the levels were much lower than those detected in protein lysates from 293 cells. The steady state E1A levels detected in protein extracts from PER cells were much higher than those detected in extracts from A549-derived cells. All PER cell lines expressed similar levels of E1A proteins (Fig. 7). The expression of the E1B proteins, particularly in the case of E1B 55 kDa, was more variable. Compared to 911 and 293, the majority of the PER clones express high levels of E1B 55 kDa and 2 kDa. The steady state level of E1B 21 kDa was the highest in PER.C3. None of the PER clones lost expression of the Ad5 E1 genes upon serial passage of the cells (not shown). We found that the level of E1 expression in PER cells remained stable for at least 100 population doublings. We decided to characterize the PER clones in more detail.

#### Southern analysis of PER clones

To study the arrangement of the Ad5-E1 encoding sequences in the PER clones we performed Southern analyses. Cellular DNA was extracted from all PER clones, and from 293 and 911 cells. The DNA was digested with *HindIII*, which cuts once in the Ad5 E1 region. Southern hybridization on *HindIII*-digested DNA, using a radiolabeled Ad5-E1-specific probe revealed the presence of several integrated copies of pIG.E1A.E1B in the genome of the PER clones. Figure 8 shows the distribution pattern of E1 sequences in the high molecular weight DNA of the different PER cell lines. The copies are concentrated in a single band, which suggests that they are integrated as tandem repeats. In the case of PER.C3, C5, C6 and C9 we found additional hybridizing bands of low molecular weight that indicate the presence of truncated copies of pIG.E1A.E1B. The number of copies was determined with the use of a Phospho-Imager. We estimated that PER.C1, C3, C4, C5, C6, C8 and C9 contain 2, 88, 5, 4, 5, 5, and 3 copies of the Ad5 E1 coding region, respectively,

and that 911 and 293 cells contain 1 and 4 copies of the Ad5 E1 sequences, respectively.

#### Transfection efficiency

5        Recombinant adenovectors are generated by co-transfection of adaptor plasmids and the large *Cla*I fragment of Ad5 into 293 cells (EP application 95202213). The recombinant virus DNA is formed by homologous recombination between the homologous viral sequences that are present in  
10    the plasmid and the adenovirus DNA. The efficacy of this method, as well as that of alternative strategies, is highly dependent on the transfectability of the helper cells. Therefore, we compared the transfection efficiencies of some of the PER clones with 911 cells, using the *E. coli*  $\beta$ -  
15    galactosidase-encoding lacZ gene as a reporter (Fig. 9).

#### Production of recombinant adenovirus

      Yields of recombinant adenovirus obtained after inoculation of 293, 911, PER.C3, PER.C5 and PER.C6 with  
20    different adenovirus vectors are presented in Table II.

      The results indicate that the recombinant adenovirus vector yields obtained with PER cells are at least as high as those obtained with the existing cell lines. In addition, the yields of the novel adenovirus vector IG.Ad.MLPI.TK are  
25    similar or higher than the yields obtained for the other viral vectors on all cell lines tested.

#### Generation of new adenovirus vectors (Fig. 10)

      The recombinant adenovirus vectors used (see patent  
30    application EP 95202213) are deleted for E1 sequences from 459 to nt. 3328. As construct pE1A.E1B contains Ad5 sequences 459 to nt. 3510 there is a sequence overlap of 183 nt. between E1B sequences in the packaging construct pIG.E1A.E1B and recombinant adenoviruses, such as for example  
35    IG.Ad.MLP.TK. The overlapping sequences were deleted from the new adenovirus vectors. In addition, non-coding

sequences derived from lacZ, that are present in the original constructs, were deleted as well. This was achieved (see Fig. 10) by PCR amplification of the SV40 poly (A) sequences from pMLP.TK using primers SV40-1 (SEQ ID NO: 33) (introduces a *Bam*HI site) and SV40-2 (SEQ ID NO: 34) (introduces a *Bgl*III site). In addition, Ad5 sequences present in this construct were amplified from nt. 2496 (Ad5, introduces a *Bgl*III site) to nt. 2779 (Ad5-2). Both PCR fragments were digested with *Bgl*III and were ligated. The ligation product was PCR amplified using primers SV40-1 and Ad5-2 (SEQ ID NO:36). The PCR product obtained was cut with *Bam*HI and *Afl*III and was ligated into pMLP.TK predigested with the same enzymes. The resulting construct, named pMLPI.TK, contains a deletion in adenovirus E1 sequences from nt. 459 to nt. 3510.

#### 15 Packaging system

The combination of the new packaging construct pIG.E1A.E1B and the recombinant adenovirus pMLPI.TK, which do not have any sequence overlap, are presented in Fig. 11. In this figure, also the original situation is presented, where the sequence overlap is indicated. The absence of overlapping sequences between pIG.E1A.E1B and pMLPI.TK (Fig. 11a) excludes the possibility of homologous recombination between the packaging construct and the recombinant virus, and is therefore a significant improvement for production of recombinant adenovirus as compared to the original situation.

In Fig. 11b the situation is depicted for pIG.E1A.NEO and IG.Ad.MLPI.TK. pIG.E1A.NEO when transfected into established cells, is expected to be sufficient to support propagation of E1-deleted recombinant adenovirus. This combination does not have any sequence overlap, preventing generation of RCA by homologous recombination. In addition, this convenient packaging system allows the propagation of recombinant adenoviruses that are deleted just for E1A sequences and not for E1B sequences.

35 Recombinant adenoviruses expressing E1B in the absence of E1A are attractive, as the E1B protein, in particular E1B



19kD, is able to prevent infected human cells from lysis by Tumor Necrosis Factor (TNF) Gooding et al, (1991) *J. Virol.* 65: 3083-3094).

5 Generation of recombinant adenovirus derived from pMLPI.TK

Recombinant adenovirus was generated by co-transfection of 293 cells with *SalI* linearized pMLPI.TK DNA and *ClaI* linearized Ad5 wt DNA. The procedure is schematically represented in Fig. 12.

10

Example 2

Plasmid-based system for rapid RCA-free generation of recombinant adenoviral vectors.

15 Construction of adenovirus clones

pBr/Ad.Bam-rITR (ECACC deposit P97082122)

In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with *BamHI*. This DNA preparation was used without further purification in a ligation reaction with pBr322 derived vector DNA prepared as follows: pBr322 DNA was digested with *EcoRV* and *BamHI*, dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LMP agarose gel (SeaPlaque GTG). After transformation into competent *E.coli* DH5a (Life Techn.) and analysis of ampicillin resistant colonies, one clone was selected that showed a digestion pattern as expected for an insert extending from the *BamHI* site in Ad5 to the right ITR. Sequence analysis of the cloning border at the right ITR revealed that the most 3' G residue of the ITR was missing, the remainder of the ITR was found to be correct. Said missing G residue is complemented by the other ITR during replication.

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pBr/Ad.Sal-rITR (ECACC deposit P97082119)

pBr/Ad.Bam-rITR was digested with *Bam*HI and *Sal*I. The vector fragment including the adenovirus insert was isolated in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb *Sal*I-  
5 *Bam*HI fragment obtained from wt Ad5 DNA and purified with the GeneClean II kit (Bio 101, Inc.). One clone was chosen and the integrity of the Ad5 sequences was determined by restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the *Sal*I site at bp 16746 up to  
10 and including the rITR (missing the most 3' G residue).

pBr/Ad.Cla-Bam (ECACC deposit P97082117)

wt Adeno type 5 DNA was digested with *Cla*I and *Bam*HI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and  
15 purified from agarose gel by GeneClean. Both fragments were ligated and transformed into competent DH5 $\alpha$ . The resulting clone pBr/Ad.Cla-Bam was analyzed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

pBr/Ad.AflIII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearized with *Eco*RI (in pBr322) and partially digested with *Afl*III. After heat inactivation of *Afl*III for 20 minutes at 65 °C, the fragment ends were filled in with Klenow enzyme. The DNA was then  
25 ligated to a blunt double stranded oligo linker containing a *Pac*I site (5'-AATTGTCTTAATTAACCGCTTAA-3') (SEQ ID NO:1). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' (SEQ ID NO:2) and 5'-AATTGCGGTTAATTAAGAC-3' (SEQ ID NO:3), followed by blunting  
30 with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess *Pac*I enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose  
35 (SeaPlaque GTG), religated and transformed into competent DH5

$\alpha$ . One clone that was found to contain the *PacI* site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the *PacI* linker in the (lost) *AflIII* site.

- 5 pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and  
pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

To allow insertion of a *PacI* site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed  
10 between the *ClaI* site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with *ClaI* and treated with nuclease *Bal31* for varying lengths of time (2', 5', 10' and 15'). The extent of nucleotide removal was followed by separate reactions on  
15 pBr322 DNA (also digested at the *ClaI* site), using identical buffers and conditions. *Bal31* enzyme was inactivated by incubation at 75 °C for 10 minutes, the DNA was precipitated and resuspended in a smaller volume TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA polymerase  
20 in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with *SalI*, satisfactory degradation (~150 bp) was observed in the samples treated for 10 minutes or 15 minutes. The 10 minutes or 15 minutes treated pBr/Ad.Bam-rITR samples were then ligated to the above  
25 described blunted *PacI* linkers (see pBr/Ad.AflIII-Bam). Ligations were purified by precipitation, digested with excess *PacI* and separated from the linkers on an LMP agarose gel. After relegation, DNAs were transformed into competent DH5 $\alpha$  and colonies analyzed. Ten clones were selected that  
30 showed a deletion of approximately the desired length and these were further analyzed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the *PacI* linker inserted just downstream of the rITR. After digestion with *PacI*, clone #2 has 28 bp and clone #8  
35 has 27 bp attached to the ITR.

pWE/Ad.AflIII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique *PacI* site was inserted in the *EcoRI* sites of pWE15 creating pWE.pac. To this end, the double stranded *PacI* oligo as described for pBr/Ad.AflIII-BamHI was used but now with its *EcoRI* protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with *PacI*, pBr/AflIII-Bam digested with *PacI* and *BamHI* and pBr/Ad.Bam-rITR#2 digested with *BamHI* and *PacI*. These fragments were ligated together and packaged using  $\lambda$  phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analyzed for presence of the complete insert. pWE/Ad.AflIII-rITR contains all adenovirus type 5 sequences from bp 3534 (*AflIII* site) up to and including the right ITR (missing the most 3' G residue).

Adeno 5 wt DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with *SalI*. Two of the resulting fragments, designated left ITR-*Sal*(9.4) and *Sal*(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was digested with *EcoRV* and *SalI* and treated with phosphatase (Life Technologies). The vector fragment was isolated using the Geneclean method (BIO 101, Inc.) and ligated to the Ad5 *SalI* fragments. Only the ligation with the 9.4 kb fragment gave colonies with an insert. After analysis and sequencing of the cloning border a clone was chosen that contained the full ITR sequence and extended to the *SalI* site at bp 9462.

pBr/Ad.lITR-*Sal*(16.7) (ECACC deposit P97082118)

pBr/Ad.lITR-*Sal*(9.4) is digested with *SalI* and dephosphorylated (TSAP, Life Technologies). To extend this clone up to the third *SalI* site in Ad5, pBr/Ad.Cla-Bam was linearized with *BamHI* and partially digested with *SalI*. A 7.3

kb *SalI* fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to the *SalI*-digested pBr/Ad.lITR-Sal(9.4) vector fragment.

pWE/Ad.AflIII-EcoRI

5 pWE.pac was digested with *ClaI* and the 5' protruding ends were filled in using Klenow enzyme. The DNA was then digested with *PacI* and isolated from agarose gel. pWE/AflIII-rITR was digested with *EcoRI* and after treatment with Klenow enzyme digested with *PacI*. The large 24 kb fragment  
10 containing the adenoviral sequences was isolated from agarose gel and ligated to the *ClaI*-digested and blunted pWE.pac vector using the Ligation Express™ kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the  
15 expected insert. pWE/AflIII-EcoRI contains Ad5 sequences from bp 3534-27336.

Construction of new adapter plasmids

The absence of sequence overlap between the recombinant  
20 adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK (fig. 10) is an example of an adapter plasmid designed for use according to the invention in combination with the improved  
25 packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily exchanged.

First, a PCR fragment was generated from pZipΔ  
30 Mo+PyF101(N<sup>-</sup>) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' (SEQ ID NO:4) and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3' (SEQ ID NO:5). Pwo DNA  
35 polymerase (Boehringer Mannheim) was used according to the

manufacturer's protocol with the following temperature cycles: once 5 minutes at 95°C; 3 minutes at 55°C; and 1 minute at 72°C, and 30 cycles of 1 minute at 95°C, 1 minute at 60°C, 1 minute at 72°C, followed by once 10 minutes at 72°C. The PCR product was then digested with *Bam*HI and ligated into a pMLP10 (Levrero et al, (1991) *Gene* 101:195-202) vector digested with *Pvu*II and *Bam*HI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter which includes part of the Mo-MuLV LTR in which the wild-type enhancer sequences are replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420.

Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with *Bst*BI followed by Klenow treatment and digestion with *Nco*I. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al, (1990) *J. Immunol.* 145:1952-1959) using the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' (SEQ ID NO:6) and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3' (SEQ ID NO:7). The 269 bp amplified fragment was subcloned in a shuttle vector using the *Nco*I and *Bgl*II sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication was then excised as a *Nco*I(sticky)-*Sal*I(blunt) fragment and cloned into the 3.5 kb *Nco*I(sticky)/*Bst*BI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with *Eco*RI and *Bam*HI after which the fragment containing the left ITR, packaging signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes, thereby replacing the promoter and the gene sequences. This resulted in the new adapter plasmid pAd/L420-HSA (Fig. 19) that contains convenient recognition sites for various restriction enzymes

around the promoter and gene sequences. *SnaBI* and *AvrII* can be combined with *HpaI*, *NheI*, *KpnI*, *HindIII* to exchange promoter sequences, while the latter sites can be combined with the *ClaI* or *BamHI* sites 3' from the HSA coding region to replace genes in this construct.

Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with *AvrII* and *BglII*, followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with *HhaI* and *AvrII* followed by treatment with T4 DNA polymerase. This adapter plasmid was named pCLIP (Fig. 20).

#### Generation of recombinant adenoviruses

##### E1-deleted recombinant adenoviruses with wt E3 sequences

To generate E1 deleted recombinant adenoviruses with the new plasmid-based system, the following constructs were prepared: an adapter construct containing the expression cassette with the gene of interest linearized with a restriction enzyme that cuts at the 3' side of the overlapping adenoviral genome fragment, preferably not containing any pBr322 vector sequences; and a complementing adenoviral genome construct pWE/Ad.AflII-rITR digested with *PacI*.

These two DNA molecules are further purified by phenol/chloroform extraction and EtOH precipitation. Co-transfection of these plasmids into an adenovirus packaging cell line, preferably a cell line according to the invention, generates recombinant replication deficient adenoviruses by a one-step homologous recombination between the adapter and the complementing construct (Fig. 21). Alternatively, instead of

pWE/Ad.AflIII-rITR other fragments can be used, e.g., pBr/Ad.Cla-Bam digested with *EcoRI* and *BamHI* or pBr/Ad.AflIII-BamHI digested with *PacI* and *BamHI* can be combined with pBr/Ad.Sal-rITR digested with *SalI*. In this case, three  
5 plasmids are combined and two homologous recombinations are needed to obtain a recombinant adenovirus (Fig. 22). It is to be understood that those skilled in the art may use other combinations of adapter and complementing plasmids without departing from the present invention.

10 A general protocol as outlined below and meant as a non-limiting example of the present invention has been performed to produce several recombinant adenoviruses using various adapter plasmids and the Ad.AflIII-rITR fragment. Adenovirus packaging cells (PER.C6) were seeded in ~25 cm<sup>2</sup> flasks and  
15 the next day when they were at ~80% confluency, were transfected with a mixture of DNA and lipofectamine agent (Life Techn.) as described by the manufacturer. Routinely, 40 µl lipofectamine, 4 µg adapter plasmid and 4 µg of the complementing adenovirus genome fragment AflIII- rITR (or 2 µg  
20 of all three plasmids for the double homologous recombination) were used. Under these conditions transient transfection efficiencies of ~50% (48 hrs post transfection) were obtained as determined with control transfections using a pAd/CMV-LacZ adapter. Two days later, cells were passaged  
25 to ~80 cm<sup>2</sup> flasks and further cultured. Approximately five (for the single homologous recombination) to eleven days (for the double homologous recombination) later a cytopathic effect (CPE) was seen, indicating that functional adenovirus has formed. Cells and medium are harvested upon full CPE and  
30 recombinant virus is released by freeze-thawing. An extra amplification step in a 80 cm<sup>2</sup> flask was routinely performed to increase the yield since at the initial stage the titers was found to be variable despite the occurrence of full CPE. After amplification, viruses was harvested and plaque  
35 purified on PER.C6 cells. Individual plaques was tested for viruses with active transgenes.



Four different recombinant adenoviruses, containing the human interleukin-3 gene (see Fig. 1, WO88/04691), the human endothelial nitric oxide gene (Janssens et al, (1992) *J. Biol. Chem.* 267:14519-14522), the Tc1A transposase gene (Vos et al, (1993) *Genes Dev.* 7:1244-1253), or the bacterial LacZ gene (Kalderon et al, (1984) *Cell* 39:499-509, have been produced using this protocol. In all cases, functional adenovirus was formed and all isolated plaques contained viruses with an active transgene.

10 E1-deleted recombinant adenoviruses with modifications in the E3 or E4 regions

Besides replacements in the E1 region it is possible to delete the E3 region or replace part of the E3 region in the adenovirus because E3 functions are not necessary for the replication, packaging and infection of a recombinant virus. This creates the opportunity to use a larger insert or to insert more than one gene without exceeding the maximum packagable size (approximately 105% of wt genome length). This can be done, for example, by deleting part of the E3 region in the pBr/Ad.Bam-rITR clone by digestion with *Xba*I and religation. This removes Ad5 wt sequences 28592-30470 including all known E3 coding regions. Another example is the precise replacement of the coding region of gp19K in the E3 region with a polylinker allowing insertion of new sequences. This leaves all other coding regions intact, obviates the need for a heterologous promoter since the transgene is driven by the E3 promoter and pA sequences, leaving more space for coding sequences and results in very high transgene expression, at least as good as in a control E1 replacement vector.

To this end, the 2.7 kb *Eco*RI fragment from wt Ad5 containing the 5' part of the E3 region was cloned into the *Eco*RI site of pBluescript (KS') (Stratagene). Next, the *Hind*III site in the polylinker was removed by digestion with *Eco*RV and *Hinc*II and subsequent religation. The resulting

clone pBS.Eco-Eco/ad5ΔHIII was used to delete the gp19K coding region. Primers 1 (5'-GGG TAT TAG GCC AAAGGCGCA-3') (SEQ ID NO:8) and 2 (5'-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3') (SEQ ID NO:9) were used to amplify a sequence  
5 from pBS.Eco-Eco/ad5ΔHIII corresponding to sequences 28511 to 28734 in wt Ad5 DNA. Primers 3 (5'-GAT CCC ATG GGG ATC CTT TAC TAA GTT ACA AAG CTA-3') (SEQ ID NO:10) and 4 (5'-GTC GCT GTA GTT GGA CTG G-3') (SEQ ID NO:11) were used on the same  
10 DNA to amplify Ad5 sequences from 29217 to 29476. The two resulting PCR fragments were ligated together by virtue of the newly introduced *NcoI* site and subsequently digested with *XbaI* and *MunI*. This fragment was then ligated into a pBS.Eco-Eco/ad5ΔHIII vector that had been partially digested with *XbaI* and *MunI*, generating pBS.Eco-Eco/ad5ΔHIII.Δgp19K.  
15 To allow insertion of foreign genes into the *HindIII* and *BamHI* site, an *XbaI* deletion was made in pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the *BamHI* sites in the Bluescript polylinker. The resulting plasmid pBS.Eco-Eco/ad5ΔHIIIΔgp19K Δ*XbaI*, contains unique *HindIII* and *BamHI* sites corresponding  
20 to sequences 28733 (*HindIII*) and 29218 (*BamHI*) in Ad5. After introduction of a foreign gene into these sites, either the deleted *XbaI* fragment is re-introduced, or the insert is re-cloned into pBS.Eco-Eco/ad5ΔHIII.Δgp19K using *HindIII* and, for example *MunI*. Using this procedure, we have generated  
25 plasmids expressing HSV-TK (McKnight (1980) *Nucl. Acid. Res.* 8:5949-5964 and Vincent et al (1996) *Hum. Gene Ther.* 7:197-205), hIL-1α (Esandi et al, (1998) *Gene Therapy* 5:xxx-yyy), rat IL-3β (Esandi et al, (1998) *Gene* 11242:xxx-yyy), luciferase (DeWit et al, (1987) *Mol. Cell Biol.* 7:725-737) or  
30 LacZ. The unique *SrfI* and *NotI* sites in the pBS.Eco-Eco/ad5ΔHIII.Δgp19K plasmid (with or without an inserted gene of interest) are used to transfer the region containing the gene of interest into the corresponding region of pBr/Ad.Bam-rITR, yielding construct pBr/Ad.Bam-rITRΔgp19K (with or without an

inserted gene of interest). This construct is used as described *supra* to produce recombinant adenoviruses. In the viral context, expression of inserted genes is driven by the adenovirus E3 promoter.

5       Recombinant viruses that are both E1 and E3 deleted are generated by a double homologous recombination procedure as described above for E1-replacement vectors using a plasmid-based system which includes: an adapter plasmid for E1 replacement according to the invention, with or without  
10       insertion of a first gene of interest, the pWE/Ad.AflIII-EcoRI fragment, and the pBr/Ad.Bam-rITRAgp19K plasmid with or without insertion of a second gene of interest.

      In a non-limiting example we describe the generation and functionality of a recombinant adenovirus containing the  
15       murine HSA gene in the E1 region and the firefly luciferase gene in the gp19K region. The luciferase gene was excised from pAd/MLP-Luc (described in EP 0707071) as a *HindIII-BamHI* construct and cloned into the *HindIII-BamHI* sites of pBS.Eco-Eco/ad5ΔHIIIAgp19KΔXbaI. Then the *MscI-MunI* fragment  
20       containing the luciferase gene was cloned into the corresponding sites of pBS.Eco-Eco/ad5Δgp19K generating pBS.Eco-Eco/ad5Δgp19K.luc. This restores the Eco-Eco fragment, but now with the luciferase gene in the place of gp19K.

25       To simplify further manipulation, the internal *EcoRI* sites in the luciferase insert were mutated without making changes to the amino acid sequence of the luciferase gene. One *EcoRI* site flanked the *HindIII* site in the 5' non-coding region of the luciferase insert and the other one was located  
30       588 bp 3' from the starting ATG. A 695 bp PCR product was generated with the following primers: 5'-CGA TAA GCT TAA TTC CTT TGT GTT T-3' (SEQ ID NO:12) and 5' -CTT AGG TAA CCC AGT AGA TCC AGA GGA GTT CAT-3' (SEQ ID NO:13) and digested with *HindIII* and *BstEII*. This fragment was then ligated to  
35       *HindIII-BstEII* digested pBS.Eco-Eco/ad5Δgp19K.luc, replacing

the corresponding insert in this vector. The resulting construct is named pBS.Eco-Eco/ad5 $\Delta$ gp19K.luc<sup>2</sup>. The luciferase gene and part of the E3 region was then excised from this clone with *SrfI* and *NotI* and introduced in the  
5 corresponding sites in pBr/Ad.Bam-rITR generating clone pBr/Ad.Bam-rITR $\Delta$ gp19K/luc<sup>2</sup>.

The adapter plasmid pAd5/S1800HSA used for the replacement of E1 in the double insert virus contains the murine HSA gene driven by a retrovirus LTR-based promoter.  
10 This adapter plasmid was generated from the pAd5/L420-HSA construct described *infra* by replacement of the promoter sequence. First a PCR product was generated on a retroviral vector based on the MFG-S vector described in WO 95/34669 using the same primers as for the amplification of the L420  
15 promoter fragment (described *infra*). This PCR amplifies the sequences corresponding to bp 453-877 in the MFG-S vector. The L420 promoter in pAd5/L420-HSA (figure 21) was then exchanged for the PCR fragment using the unique *AvrII* and *HindIII* sites. The resulting construct, pAd5/S430-HSA, was  
20 then digested with *NheI* and *ScaI* and the 4504 bp fragment containing the HSA gene, pA sequences, Ad5 sequences and vector sequences to the *ScaI* site in the ampicillin gene was isolated.

The construct pAd5/S430-HSA also was digested with *XbaI*  
25 and *ScaI* and the 1252 bp fragment (containing the remainder of the ampicillin gene, the left ITR and packaging signal from adenovirus and the 5' part of the S430 promoter) was isolated. A third fragment of 1576 bp was isolated from the MFG-S-based retroviral vector following an *XbaI* digestion and  
30 contains MFG-S sequences corresponding to bp 695-2271.

The adapter plasmid pAd5/S1800-HSA was constructed by ligating the three isolated fragments. The double insert virus Ad5/S1800-HSA.E3luc was generated (as described above) by transfection of the following DNA fragments into PER.C6  
35 cells: pAd5/S1800-HSA digested with *EcoRI* and *SalI* (2  $\mu$ g).

At occurrence of CPE, the virus was harvested and amplified by serial passages on PER.C6 cells. The activity of this HSA-luc virus was compared to single insert  $\Delta$ E1 viruses containing either the S1800-HSA or the CMV-luc transcription units in the E1 region. A549 cells were seeded at  $2 \times 10^5$  cells/well and infected 5 hrs later with different amounts of the virus. Two days later transgene expression was measured. Luciferase activity was measured using a luciferase assay system (Promega) and expression of the murine HSA gene was measured with an  $\alpha$ -HSA antibody (M1/69, Pharmingen). The results are listed in Table III.

This experiment shows that using the plasmid-based recombination system, double insert viruses can be made and that both inserts are functional. Furthermore, the luciferase activity of the double insert viruses is comparable to the CMV-driven luciferase activity of the control virus. Therefore, we conclude that the E3 promoter is highly active in A549 cells, even in the absence of E1A proteins.

In addition to manipulations in the E3 region, changes of (parts of) the E4 region can be accomplished easily in pBr/Ad.Bam-rITR. Generation and propagation of such a virus, however, in some cases demands complementation *in trans*.

### Example 3

Demonstration of the competence of a synthetic DNA sequence, that is capable of forming a hairpin structure, to serve as a primer for reverse strand synthesis for the generation of double-stranded DNA molecules in cells that contain and express adenovirus genes.

Name convention of the plasmids used:

- p plasmid
- I ITR (Adenovirus Inverted Terminal Repeat)
- C Cytomegalovirus (CMV) Enhancer/Promoter Combination
- L Firefly Luciferase Coding Sequence
- hac, haw Potential hairpin that can be formed after digestion with restriction endonuclease Asp718 in

both the correct and in the reverse orientation, respectively (Fig. 15)

The naming convention is exemplified as follows.

pICLhaw is a plasmid that contains the adenovirus ITR followed by the CMV-driven luciferase gene and the Asp718 hairpin in the reverse (non-functional) orientation. Plasmids pICLhac, pICLhaw, pICLI and pICL were generated using standard techniques. The schematic representation of these plasmids is shown in Figs. 16-19.

Plasmid pICL is derived from the following plasmids:

nt.1	457 pMLP10 (Levrero et al, (1991) Gene 101:195-202)
nt.458	1218 pCMV $\beta$ (Clontech, EMBL Bank No. U02451)
nt.1219	3016 pMLP.luc (IntroGene, unpublished)
nt.3017	5620 pBLCAT5 (Stein et al, (1989) Mol. Cell Biol. 9:4531-4).

The plasmid has been constructed as follows:

The tet gene of plasmid pMLP10 has been inactivated by deletion of the *Bam*HI-*Sal*I fragment, to generate pBLP10 $\Delta$ SB. Using primer set PCR/MLP1 (SEQ ID NO:37) and PCR/MLP3 (SEQ ID NO:38) a 210 bp fragment containing the Ad5-ITR, flanked by a synthetic *Sal*I restriction site was amplified using pMLP10 DNA as the template. The PCR product was digested with the enzymes *Eco*RI and *Sgr*AI to generate a 196 bp fragment. Plasmid pMLP10 $\Delta$ SB was digested with *Eco*RI and *Sgr*AI to remove the ITR. This fragment was replaced by the *Eco*RI-*Sgr*AI-treated PCR fragment to generate pMLP/SAL.

Plasmid pCMV-Luc was digested with *Pvu*II to completion and recirculated to remove the SV40-derived poly-adenylation signal and Ad5 sequences with exception of the Ad5 left-terminus. In the resulting plasmid, pCMV-luc $\Delta$ Ad, the Ad5 ITR was replaced by the *Sal*-site-flanked ITR from plasmid pMLP/SAL by exchanging the *Xmn*I-*Sac*II fragments. The resulting plasmid, pCMV-luc $\Delta$ Ad/SAL, the Ad5 left terminus and

the CMV-driven luciferase gene were isolated as a *SalI-SmaI* fragment and inserted in the *SalI* and *HpaI* digested plasmid pBLCATS, to form plasmid pICL. Plasmid pICL is represented in Fig. 19; its sequence is presented in Fig. 20.

5 Plasmid pICL contains the following features:

nt.1-457 Ad5 left terminus (Sequence 1-457 of human adenivorus type 5)

nt.458-969 Human cytomegalovirus enhancer and immediate early promoter (Boshart et al, (1985) Cell 41:521-530) (from plasmid pCMV $\beta$ , Clontech, Palo Alto, USA)

nt.970-1204 SV40 19S exon and truncated 16/19S intron (from plasmid pCMV $\beta$ )

nt.1218-2987 Firefly luciferase gene (from pMLP.luc)

15 nt.3018-3131 SV40 tandem poly-adenylation signals from late transcript,

derived from plasmid pBLCAT5)

nt.3132-5620 pUC12 backbone (derived from plasmid pBLCAT5)

20 nt.4337-5191  $\beta$ -lactamase gene (Amp-resistance gene, reverse orientation)

#### Plasmids pICLhac and pICLhaw

Plasmids pICLhac and pICLhaw were derived from plasmid pICL by digestion of pICL with the restriction enzyme *Asp718*. The linearized plasmid was treated with Calf-Intestine Alkaline Phosphatase to remove the 5' phosphate groups. The partially complementary synthetic single-stranded oligonucleotides Hp/aspl (SEQ ID NO:39) and Hp/asp2 (SEQ ID NO:40) were annealed and phosphorylated on their 5' ends using T4-polynucleotide kinase.

The phosphorylated double-stranded oligomers were mixed with the dephosphorylated pICL fragment and ligated. Clones containing a single copy of the synthetic oligonucleotide inserted into the plasmid were isolated and characterized using restriction enzyme digests. Insertion of the

oligonucleotide into the *Asp*718 site will at one junction recreate an *Asp*718 recognition site, whereas at the other junction the recognition site will be disrupted. The orientation and the integrity of the inserted oligonucleotide was verified in selected clones by sequence analyses. A clone containing the oligonucleotide in the correct orientation (the *Asp*718 site close to the 3205 *Eco*RI site) was denoted pICLhac. A clone with the oligonucleotide in the reverse orientation (the *Asp*718 site close to the SV40 derived poly signal) was designated pICLhaw. Plasmids pICLhac and pICLhaw are represented in Figs. 16 and 17.

Plasmid pICLI was created from plasmid pICL by insertion of the *Sal*I-*Sgr*AI fragment from pICL, containing the Ad5-ITR into the *Asp*718 site of pICL. The 194 bp *Sal*I-*Sgr*AI fragment was isolated from pICL, and the cohesive ends were converted to blunt ends using *E. coli* DNA polymerase I (Klenow fragment) and dNTP's. The *Asp*718 cohesive ends were converted to blunt ends by treatment with mungbean nuclease. By ligation clones were generated that contain the ITR in the *Asp*718 site of plasmid pICL. A clone that contained the ITR fragment in the correct orientation was designated pICLI (Fig. 18).

Generation of adenovirus Ad-CMV-hcTK. Recombinant adenovirus was constructed according to the method described in Patent application 95202213. Two components are required to generate a recombinant adenovirus. First, an adaptor-plasmid containing the left terminus of the adenovirus genome containing the ITR and the packaging signal, an expression cassette with the gene of interest, and a portion of the adenovirus genome which can be used for homologous recombination. In addition, adenovirus DNA is needed for recombination with the aforementioned adaptor plasmid. In the case of Ad-CMV-hcTK, the plasmid PCMV.TK was used as a basis. This plasmid contains nt.1-455 of the adenovirus type 5 genome, nt. 456-1204 derived from pCMV $\beta$  (Clontech, the *Pst*I-*Stu*I fragment that contains the CMV enhancer promoter



and the 16S/19S intron from simian Virus 40), the Herpes Simplex Virus thymidine kinase gene (described in EP patent application 95202213.5), the SV40-derived polyadenylation signal (nt. 2533-2668 of the SV40 sequence), followed by the

5 BglIII-ScaI fragment of Ad5 (nt. 3328-6092 of the Ad5 sequence). These fragments are present in a pMLP10-derived (Levrero et al, (1991) *Gene* 101:195-202) backbone. To generate plasmid pAD-CMVhc-TK, plasmid pCMV.TK was digested with ClaI (the unique ClaI-site is located just upstream of

10 the TK open reading frame) and dephosphorylated with Calf-Intestine Alkaline Phosphate. To generate a hairpin-structure, the synthetic oligonucleotides HP/c1a1 (SEQ ID NO:41) and HP/c1a2 (SEQ ID NO:42) were annealed and phosphorylated on their 5' -OH groups with T4-polynucleotide

15 kinase and ATP. The double-stranded oligonucleotide was ligated with the linearized vector fragment and used to transform *E. coli* strain Sure. Insertion of the oligonucleotide into the ClaI site will disrupt the ClaI recognition sites. The oligonucleotide contains a new ClaI

20 site near one of its termini. In selected clones, the orientation and the integrity of the inserted oligonucleotide was verified by sequence analyses. A clone containing the oligonucleotide in the correct orientation (the ClaI site at the ITR side) was denoted pAd-CMV-hcTK. This plasmid was co-

25 transfected with ClaI-digested wild-type adenovirus-type5 DNA into 911 cells. A recombinant adenovirus in which the CMV-hcTK expression cassette replaces the E1 sequences was isolated and propagated using standard procedures.

To study whether the hairpin can be used as a primer for

30 reverse strand synthesis on the displaced strand after replication has started at the ITR, the plasmid pICLhac was introduced into 911 cells, i.e. human embryonic retinoblasts transformed with the adenovirus E1 region. The plasmid pICLhaw served as a control: it contains the oligonucleotide

35 pair HP/asp 1 (SEQ ID NO:39) and 2 (SEQ ID NO:40) in the

reverse orientation but is otherwise completely identical to plasmid pICLhac. Also included in these studies were plasmids pICLI and pICL. In the plasmid pICLI the hairpin is replaced by an adenovirus ITR. Plasmid pICL contains neither  
5 a hairpin nor an ITR sequence. These plasmids served as controls to determine the efficiency of replication by virtue of the terminal hairpin structure. To provide the viral products other than the E1 proteins (these are produced by the 911 cells) required for DNA replication the cultures were  
10 infected with the virus IG.Ad.MLPI.TK after transfection. Several parameters were being studied to demonstrate proper replication of the transfected DNA molecules. First, DNA extracted from the cell cultures transfected with the aforementioned plasmids and infected with IG.Ad.MLPI.TK virus  
15 was analyzed by Southern blotting for the presence of the expected replication intermediates, as well as for the presence of the duplicated genomes. Furthermore, from the transfected and IG.Ad.MLPI.TK infected cell populations, virus was isolated that can transfer a luciferase marker gene  
20 into luciferase negative cells and express it.

Plasmid DNA of plasmids pICLhac, pCLhaw, pICLI and pICL were digested with restriction endonuclease *SalI* and treated with mungbean nuclease to remove the 4 nucleotide single-stranded extension of the resulting DNA fragment. In this  
25 manner a natural adenovirus 5' ITR terminus on the DNA fragment was created. Subsequently, both the pICLhac and pICLhaw plasmids were digested with restriction endonuclease *Asp718* to generate the terminus capable of forming a hairpin structure. The digested plasmids were introduced into 911  
30 cells, using the standard calcium phosphate co-precipitation technique, four dishes for each plasmid. During the transfection, for each plasmid two of the cultures were infected with the IG.Ad.MLPI.TK virus using 5 infectious IG.Ad.MLPI.TK particles per cell. At twenty-hours post  
35 transfection and forty hours post-transfection one Ad.tk-virus-infected and one uninfected culture were used to

isolate low molecular-weight DNA using the procedure devised by Hirt (as described in Einerhand et al, (1995) *Gene Therapy* 2:336-343). Aliquots of isolated DNA were used for Southern analysis. After digestion of the samples with restriction endonuclease *EcoRI* using the luciferase gene as a probe a hybridizing fragment of approx. 2.6kb were detected in only the samples from the adenovirus-infected cells transfected with plasmid pICLhac. The size of this fragment was consistent with the anticipated duplication of the luciferase marker gene. This supports the conclusion that the inserted hairpin is capable of serving as a primer for reverse strand synthesis. The hybridizing fragment was absent if the IG.Ad.MLPI.TK virus was omitted, or if the hairpin oligonucleotide was inserted in the reverse orientation.

The restriction endonuclease *DpnI* recognizes the tetranucleotide sequence 5'-GATC-3', but cleaves only methylated DNA, (that is, only plasmid DNA propagated in, and derived, from *E. coli*, not DNA that has been replicated in mammalian cells). The restriction endonuclease *MboI* recognizes the same sequences, but cleaves only unmethylated DNA (namely, DNA propagated in mammalian cells). DNA samples isolated from the transfected cells are incubated with *MboI* and *DpnI* and analyzed with Southern blots. These results demonstrated that only in the cells transfected with the pICLhac and the pICLI plasmids large *DpnI*-resistant fragments were present, that were absent in the *MboI* treated samples. These data demonstrate that only after transfection of plasmids pICLI and pICLhac replication and duplication of the fragments occur.

These data demonstrate that in adenovirus-infected cells linear DNA fragments that have on one terminus an adenovirus-derived inverted terminal repeat (ITR) and at the other terminus a nucleotide sequence that can anneal to sequences on the same strand, when present in single-stranded form thereby generate a hairpin structure, and will be converted

to structures that have inverted terminal repeat sequences on both ends. The resulting DNA molecules will replicate by the same mechanism as the wild-type adenovirus genomes.

5

#### Example 4

Demonstration that the DNA molecules that contain a luciferase marker gene, a single copy of the ITR, the encapsidation signal and a synthetic DNA sequence, that is capable of forming a hairpin structure, are sufficient to generate DNA molecules that can be encapsidated into virions.

To demonstrate that the DNA molecules, generated in Example 3, containing two copies of the CMV-luc marker gene can be encapsidated into virions, virus was harvested from the remaining two cultures via three cycles of freeze-thaw crushing and was used to infect murine fibroblasts. Forty-eight hours after infection the infected cells are assayed for luciferase activity. To exclude the possibility that the luciferase activity has been induced by transfer of free DNA, rather than via virus particles, virus stocks were treated with DNaseI to remove DNA contaminants. Furthermore, as an additional control, aliquots of the virus stocks were incubated for 60 minutes at 56°C. The heat treatment does not affect the contaminating DNA, but does inactivate the viruses. Significant luciferase activity was only found in the cells after infection with the virus stocks derived from IG.Ad.MLPI.TK-infected cells transfected with the pICLhc and pICLI plasmids. Neither in the non-infected cells, nor in the infected cells transfected with the pICLhw and pICL was significant luciferase activity demonstrated. Heat inactivation, but not DNaseI treatment, completely eliminated luciferase expression, demonstrating that adenovirus particles, and not free (contaminating) DNA fragments were responsible for transfer of the luciferase reporter gene.

These results demonstrate that these small viral genomes can be encapsidated into adenovirus particles and suggest that the ITR and the encapsidation signal are sufficient for encapsidation of linear DNA fragments into adenovirus

particles. These adenovirus particles can be used for efficient gene transfer. When introduced into cells that contain and express at least some of the adenovirus genes (namely E1, E2, E4, and L, and VA), recombinant DNA molecules that include at least one ITR, at least part of the encapsidation signal as well as a synthetic DNA sequence, that is capable of forming a hairpin structure, have the intrinsic capacity to autonomously generate recombinant genomes which can be encapsidated into virions. Such genomes and vector system can be used for gene transfer.

#### Example 5

Demonstration that DNA molecules which contain nucleotides 3510-35953 (namely 9.7-100 map units) of the adenovirus type 5 genome (thus lack the E1 protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion of the same strand of the DNA molecule when present in single-stranded form other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells.

In order to develop a replicating DNA molecule that can provide the adenovirus products required to allow the above-mentioned ICLhac vector genome and alike minimal adenovectors to be encapsidated into adenovirus particles by helper cells, the Ad-CMV-hcTK adenoviral vector was developed. Between the CMV enhancer/promoter region and the thymidine kinase gene, the annealed oligonucleotide pair (Table I) HP/cia 1 and 2 was inserted. The vector Ad-CMV-hcTK was propagated and produced in 911 cell using standard procedures. This vector was grown and propagated exclusively as a source of DNA used for transfection. DNA of the adenovirus Ad-CMV-hcTK was isolated from virus particles that had been purified using CsCl density-gradient centrifugation by standard techniques. The virus DNA was digested with restriction endonuclease ClaI. The digested DNA was size-fractionated on an 0.7% agarose gel and the large fragment was isolated and used for further experiments. Cultures of 911 cells were transfected with the large ClaI-fragment of the Ad-CMV-hcTK DNA using

standard calcium phosphate co-precipitation techniques. Much like in the previous experiments with plasmid pICLhac, the Ad-CMV-hc replicates starting at the right-hand ITR. Once the 1-strand is displaced, a hairpin can be formed at the left-hand terminus of the fragment. This facilitates DNA polymerase elongation of the chain towards the right-hand side. The process proceeds until the displaced strand is completely converted to its double-stranded form. Finally, the right-hand ITR is recreated, and in this location, normal adenovirus replication-initiation and elongation occur. The polymerase reads through the hairpin, thereby duplicating the molecule. The input DNA molecule of 33250 bp, that had on one side an adenovirus ITR sequence and at the other side a DNA sequence that had the capacity to form a hairpin structure is duplicated so that both ends contain an ITR sequence. The resulting DNA molecule consists of a palindromic structure of approximately 66500 bp.

This structure is detected in low-molecular weight DNA extracted from transfected cells using Southern analysis. The palindromic nature of the DNA fragment can be demonstrated by digestion of the low-molecular weight DNA with suitable restriction endonucleases and Southern blotting with the HSV-TK gene as the probe. This molecule can replicate itself in the transfected cells by virtue of the adenovirus gene products that are present in the cells. In part, the adenovirus genes are expressed from templates that are integrated in the genome of the target cells (namely, the E1 gene products), the other genes reside in the replicating DNA fragment itself. This linear DNA fragment cannot be encapsidated into virions. Not only does it lack all the DNA sequences required for encapsidation, but its size also is much too large to be encapsidated.

**Example 6**

Demonstration that DNA molecules which contain nucleotides 3503-35953 (viz. 9.7-100 map units) of the adenovirus type 5 genome (thus lack the E1 protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion the same strand of the DNA molecule other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells and can provide the helper functions required to encapsidate the pICLI and pICLhac derived DNA fragments.

The purpose of the next series of experiments is to demonstrate that the DNA molecule described in Example 5 can be used to encapsidate the minimal adenovectors described in Examples 3 and 4.

The large fragment isolated after endonuclease *Cla*I-digestion of Ad-CMV-hcTK DNA was introduced into 911 cells (as described in Example 5) together with endonuclease *Sal*I, mungbean nuclease, endonuclease *Asp*718-treated plasmid pICLhac, or as a control similarly treated plasmid pICLhaw. After 48 hours virus was isolated by freeze-thaw crushing of the transfected cell population. The virus preparation was treated with DNaseI to remove contaminating free DNA. The virus was used subsequently to infect Rat2 fibroblasts. Forty-eight hours post infection the cells were assayed for luciferase activity. Only in the cells infected with virus isolated from the cells transfected with the pICLhac plasmid, and not with the pICLhaw plasmid, was significant luciferase activity demonstrated. Heat inactivation of the virus prior to infection completely abolished the luciferase activity, indicating that the luciferase gene was transferred by a viral particle. Infection of 911 cell with the virus stock did not result in any cytopathological effects, demonstrating that pICLhac was produced without any infectious helper virus being propagated on 911 cells. These results demonstrate that the proposed method can be used to produce stocks of minimal-adenoviral vectors, that are completely devoid of infectious helper viruses that are able to replicate

autonomously on adenovirus-transformed human cells or on non-adenovirus transformed human cells.

#### Example 7

##### 5        Construction of plasmids for the generation and production of minimal adenoviral vectors.

A minimal adenovirus vector contains as operably linked components the adenovirus-derived *cis* elements necessary for  
10 replication and packaging, with or without foreign nucleic acid molecules to be transferred. Recently, the lower limit for efficient packaging of adenoviral vectors has been determined at 75% of the genome length (Parks and Graham, 1997). To allow flexible incorporation of various lengths of  
15 stuffer fragments, a multiple cloning site (MCS) was introduced into a minimal adenoviral vector. To obtain a minimal adenoviral vector according to the invention, the following constructs were made: pAd/L420-HSA (Fig. 19) was digested with *Bgl*III and *Sal*I and the vector-containing  
20 fragment was isolated. This fragment contains the left ITR and packaging signal from Ad5 and the murine HSA gene driven by a modified retroviral LTR. The right ITR of adenovirus was amplified by PCR on pBr/Ad.BamHI-rITR template DNA using the following primers: PolyL-ITR: 5'-AAC-TGC-AGA-TCT-ATC-GAT-ACT-  
25 AGT-CAA-TTG-CTC-GAG-TCT-AGA-CTA-CGT-CAC-CCG-CCC-CGT-TCC-3' (SEQ ID NO:14) and ITR-BSN: 5'-CGG-GAT-CCG-TCG-ACG-CGG-CCG-CAT-CAT-CAA-TAA-TAT-ACC-3' (SEQ ID NO:15). The amplified fragment was digested with *Pst*I and *Bam*HI and cloned into pUC119 digested with the same enzymes. After sequence  
30 confirmation of correct amplification of the ITR and the MCS, a *Bgl*III-*Sal*I fragment was isolated and cloned into the *Bgl*III/*Sal*I-digested pAd/L420-HSA fragment described above. The resulting clone was named pAd/L420-HSA.ITR.

To be able to manipulate constructs of lengths exceeding  
35 30 kb, the minimal adenoviral vector pAd/L420-HSA.ITR was subcloned in a cosmid vector background. To this end, the cosmid vector pWE15 was modified to remove restriction sites



in the backbone. pWE15 was digested with *Pst*I and fragments of 4 kb and 2,36 kb were isolated from agarose gel and ligated together. The resulting clone, stripped of the SV40 *ori*/early promoter and neomycine resistance coding sequence, was named pWE20. Then, pWE20 was digested with *Cla*I and *Hind*III and the sticky ends were filled in with Klenow enzyme. A 6354 bp blunt fragment was ligated to a phosphorylated *Nsi*I linker with the following sequence: 5'-CGATGCATCG-3' (SEQ ID NO:16). The ligated DNA was phenol/chloroform extracted, precipitated with EtOH to change buffers, and digested with excess *Nsi*I. Digested DNA was separated from the linkers by electrophoresis, isolated and religated. The resulting clone was named pWE25. Correct insertion of the *Nsi*I linker was confirmed by restriction enzyme digestion and sequencing. To construct the minimal adenoviral vector, pAd/L420-HSA.ITR was digested with *Sca*I and *Not*I and the 2 kb fragment containing part of the ampicillin gene and the adeno ITRs was cloned into pWE25 digested with *Sca*I and *Not*I. The resulting clone was named pMV/L420H (Fig. 24). This clone allows easy manipulation to exchange the promoter and/or gene, and also allows insertion of DNA fragments of lengths not easily cloned into normal plasmid backbones.

Plasmid pMV/CMV-LacZ was made by exchanging the L420-HSA fragment (*Sna*BI-*Bam*HI) for a fragment from pCDNA3-nlsLacZ (*Nru*I-*Bam*HI) containing the CMV promoter and LacZ coding sequences. pCDNA3-nlsLacZ was constructed by insertion of a *Kpn*I-*Bam*HI fragment obtained after PCR amplification of the nlsLacZ coding sequences into pCDNA3 (Invitrogen) digested with *Kpn*I and *Bam*HI. The PCR reaction was performed on a pMLP.nlsLacZ template DNA using the primers 1: 5'-GGG-GTG-GCC-AGG-GTA-CCT-CTA-GGC-TTT-TGC-AA-3' (SEQ ID NO:17) and 2: 5'-GGG-GGG-ATC-CAT-AAA-CAA-GTT-CAG-AAT-CC-3' (SEQ ID NO:18). Correct amplification and cloning were confirmed by assaying

$\beta$ -galactosidase expression in a transient transfection experiment on 911 cells.

The vector pAd/MLPnlsLacZ was made as follows: pMLP10 (Levrero et al, (1991) Gene 101: 195-202) was digested with  
5 *Hind*III and *Bam*HI and ligated, in a three-part ligation, to a  
3.3 kb *Avr*II-*Bam*HI fragment from L7RHbgal (Kalderon et al,  
(1984) Cell 499-509), and a synthetic linker with *Hind*III and  
*Xba*I overhang. The linker was made by annealing two  
oligonucleotides of sequence 5'-AGC TTG AAT TCC CGG GTA CCT-  
10 3' (SEQ ID NO:19) and 5'-CTA GAG GTA CCC GGG AAT TCA-3' (SEQ  
ID NO:20). The resulting clone was named pMLP.nlsLacZ/-Ad.  
Next, pMLP.nlsLacZ/-Ad was digested with *Bam*HI and *Nru*I and  
the vector containing fragment was ligated to a 2766 bp  
*Bgl*III-*Sca*I fragment from pAd5SalB (Bernards et al, (1982)  
15 *Virology* 120:422-432). This resulted in the adapter plasmid  
pMLP.nlsLacZ (described in EP 0 707 071).

Propagation of a minimal adenoviral vector can only be  
achieved by expression of adenovirus gene products.  
Expression of adenovirus gene products, at levels high enough  
20 to sustain production of large quantities of virus, requires  
replication of the coding nucleic acid molecule. Usually,  
therefore, replicating helper viruses are used to complement  
the minimal adenoviral vectors. The present invention,  
however, provides packaging systems for minimal adenoviral  
25 vectors without the use of helper viruses. One of the  
methods of the invention makes use of a replicating DNA  
molecule that contains the 5'-ITR and all adenoviral  
sequences between bp 3510 and 35938, i.e., the complete  
adenoviral genome except for the E1 region and the packaging  
30 signal. Construct pWE/Ad. $\Delta$ 5' (fig 23) is an example of a  
replicating molecule according to the invention that contains  
two adenoviral ITRs. pWE/Ad. $\Delta$ 5'. It has been made in a  
cosmid vector background from three fragments. First, the 5'  
ITR from Ad5 was amplified using the following primers:

ITR-EPH: 5'-CGG-AAT-TCT-TAA-TTA-AGT-TAA-CAT-CAT-CAA-TAA-TAT-ACC-3' (SEQ ID NO:21) and ITR-pIX: 5'-ACG-GCG-CGC-CTT-AAG-CCA-CGC-CCA-CAC-ATT-TCA-GTA-CGT-ACT-AGT-CTA-CGT-CAC-CCG-CCC-CGT-TCC-3' (SEQ ID NO:22). The resulting PCR fragment was  
5 digested with *EcoRI* and *AscI* and cloned into vector pNEB193 (New England Biolabs) digested with the same enzymes. The resulting construct was named pNEB/ITR-pIX. Sequencing confirmed correct amplification of the Ad5 sequences in the left ITR (Ad5 sequences 1 to 103) linked to the pIX promoter  
10 (Ad5 sequences 3511 to 3538) except for a single mismatch with the expected sequence according to GenBank (Accession no.: M73260/M29978), i.e., an extra C-residue was found just upstream of the *AflIII* site. This ITR-pIX fragment was isolated with *EcoRI* and *AflIII* and ligated to a *EcoRI*-*AflIII*  
15 vector fragment containing Ad5 sequences 3539-21567. The latter fragment was obtained by digestion of pBr/Ad.Cla-Bam (*supra*) with *EcoRI* and partially with *AflIII*. The resulting clone was named pAd/LITR( $\Delta$ 5')-BamHI. The final construct pWE/Ad. $\Delta$ 5' was made by ligating cosmid vector pWE15.Pac  
20 (*supra*) digested with *PacI* to pAd/LITR( $\Delta$ 5')-BamHI digested with *PacI*/BamHI and pBr/Ad.Bam-rITR.pac#2 (*supra*) digested with *PacI*/BamHI (Fig. 23).

An alternative method to produce packaging systems for minimal adenoviral vectors without the use of helper viruses  
25 according to the invention is to use a replicating DNA molecule that contains the complete adenoviral genome except for the E1 region and the packaging signal and in which one of the ITRs is replaced by a fragment containing a DNA sequence complementary to a portion of the same strand other  
30 than the ITR and that therefore is able to form a hairpin structure (Fig 10). In a non-limiting example, said DNA sequence complementary to a portion of the same strand other than the ITR is derived from the adeno-associated virus (AAV) terminal repeat. Such a replicating DNA molecule is made  
35 following the same cloning strategy as described for pWE/Ad. $\Delta$

5', but now starting with the AAV terminal repeat linked to part of the adenoviral pIX promoter. To this end, the adenoviral ITR sequences between the *HpaI* and *SpeI* sites in construct pNEB/ITR-pIX were exchanged for the AAV ITR by introducing the *PvuII/XbaI* fragment from psub201(+) containing the AAV ITR (Samulski *et al*, (1989) *J. Virol.* 63:3822-3828). This results in construct pWE/AAV.Δ5' that replicates in an E1 complementing cell line.

Another alternative packaging system for minimal adenoviral vectors is described *infra* and makes use of the replication system of SV40. A functional helper molecule according to this method contains at least the adenoviral sequences necessary to sustain packaging of a minimal construct but not the E1 sequences and packaging signal, and preferably also lacking ITRs. This adenovirus-derived entity has to be present on a vector that contains, besides the sequences needed for propagation in bacteria, an origin of replication from SV40 virus. Transfection of such a molecule together with the minimal adenoviral vector, described *supra*, into a packaging cell line (e.g. PER.C6) expressing, besides the E1 proteins, SV40 derived Large T antigen proteins, results in Large T-dependent replication of the adenovirus-derived helper construct. This replication leads to high levels of adenoviral proteins necessary for replication of the minimal adenoviral vector and packaging into virus particles. In this way, there is no sequence overlap that leads to homologous recombination between the minimal adenoviral vector construct and the helper molecule. In addition, there is no sequence overlap that leads to homologous recombination between the helper molecule and minimal adenoviral vector on the one side and the E1 sequence in the packaging cell on the other side.

Replication of a 40 kb adenoviral construct was investigated in cells expressing SV40 Large T proteins. Hereto,  $2 \times 10^6$  Cos-1 cells were transfected in a T25 flask with the following constructs complexed with lipofectamine

reagent (Life techn.): the 8 kb cosmid vector pWE.pac, the 40.5 kb construct pWE/Ad.AflIII-rITR and three clones (#1, #5 and #9) of the 40.6 kb construct pWE/Ad.Δ5' (described *infra*). Control transfections were carried out with the

5 constructs pWE.pac and pWE/Ad.AflIII-rITR digested with *PacI* enzyme and a CMV-LacZ expression vector without the SV40 *ori* sequence. Transfection efficiency was 50% as determined by a separate transfection using the CMV-LacZ vector and X-gal staining after 48 hrs. All cells were harvested 48 hrs.

10 following transfection and DNA was extracted according to the Hirt procedure (as described in Einerhand *et al*, (1995) *Gene Therapy* 2:336-343). Final pellets were resuspended in 50μl TE+RNase (20 μg/ml) and 10 μl samples were digested with *MboI* (35 units overnight at 37°C). Undigested samples (5μl) and

15 *MboI* digested samples were run on a 0.8% agarose gel, transferred to a nylon filter (Amersham) and hybridized to radioactive probes according to standard procedures. One probe was derived from an 887 bp *DpnI* fragment from the cosmid vector pWE.pac and one was derived from a 1864 bp

20 *BsrGI-BamHI* fragment from adenoviral sequences. These probes hybridize to a 887 bp band and a 1416 bp respectively in *MboI* digested material. Input DNA from bacterial origin is methylated and therefore not digested with *MboI*. In this way it is possible to specifically detect DNA that is replicated

25 in eukaryotic cells. Figure 26A shows a schematic presentation of the construct pWE/Ad.Δ5' and the locations of the SV40 origin of replication, the pWE-derived probe and the adenovirus-derived probe. The lower part presents the autoradiograms of the Southern blots hybridized to the

30 adenovirus probe (B) and the pWE probe (C). See legends for explanation of sample loading. These experiments show that all lanes that contain material from Cos-1 cells that are transfected with plasmids harbouring an SV40 *ori* contain *MboI* sensitive DNA and show a specific band of the expected

35 length. The bands specific for replication in the lanes with

Cos-1 cells transfected with *PacI* digested material (lanes B17/18 and C 15-18) probably result from incomplete *PacI* digestion. From these experiments it can be concluded that it is possible to replicate large DNA fragments with the SV40 LargeT/*ori* system in eukaryotic cells.

### Example 8

A functional adenovirus helper molecule lacking ITR sequences was constructed starting with the clone pWE/Ad.D5' described *supra*. pWE/Ad.D5' was digested with *Bst*1107I and the 17.5 kb vector-containing fragment was religated to give pWE/Ad.D5'-*Bst*1107I. This clone was then used to amplify the 3' part of the adenovirus genome sequences without the right ITR. A 2645 bp PCR fragment was generated using the primers Ad3'/Forw: 5'-CGG AAT TCA TCA GGA TAG GGC GGT GG-3' (SEQ ID NO:23) and Ad3'/Rev: 5'-CGG GAT CCT ATC GAT ATT TAA ATG TTT TAG GGC GGA GTA ACT TG-3' (SEQ ID NO:24). The amplified fragment was digested with *EcoRI* and *Bam*HI and subcloned in pBr322 digested with the same enzymes. After confirmation of correct amplification by sequencing, the 2558 bp *Sbf*I-*Cla*I fragment of this clone was recloned in pWE/Ad.D5'-*Bst*1107I digested with the same enzymes. The resulting construct lacks the right ITR and is named pWE/ $\Delta$ R1-*Bst*1107I. Next, in this clone the left ITR was replaced by a linker with a *PacI* and *Afl*III overhang made up by annealing the following primers: PA-pIX1 5'-TAA GCC ACT AGT ACG TAC TGA AAT GTG TGG GCG TGG C-3' (SEQ ID NO:25) and PA-pIX2 5'-TTA AGC CAC GCC CAC ACA TTT CAG TAC GTA CTA GTG GCT TAA T-3' (SEQ ID NO:26). This removed the left ITR and restored correct sequence of the pIX promoter. The clone is named pWE/ $\Delta$ ITR-*Bst*1107I. Correct insertion of the double stranded linker was confirmed by sequencing. The deleted *Bst*1107I fragment was then cloned back into pWE/ $\Delta$ ITR-*Bst*1107I and the correct orientation was checked by restriction digestion. The resulting clone is named pWE/Ad-H. Following transfection of this DNA molecule

into packaging cells that express adenoviral E1 proteins and the SV40 Large T antigen, replication of that molecule takes place resulting in high levels of adenoviral proteins encoded by the adenoviral entity on that molecule.

5

### Example 9

#### Miniaturized, multiwell production of recombinant adenoviral vectors

10 A 96-well microtiter tissue culture plate (plate 1)  
(Greiner, The Netherlands, catalogue #6555180) was first  
coated with poly-L-lysine (PLL, 0.1 mg/ml) (Sigma) dissolved  
in sterile water by incubating each well for 20-120 minutes  
at room temperature. Alternatively, precoated 96-well plates  
15 can be used (Becton and Dickinson). After the incubation  
with PLL, each well was washed two times with 100 µl sterile  
water and dried at room temperature for at least two hours.  
The day before transfection PER.C6 cells were harvested using  
trypsin-EDTA and counted. The cells were then diluted to a  
20 suspension of 45,000 cells per 100 µl followed by seeding 100  
µl per well of the PLL coated 96-well plates. The next day  
2.6 µl of Sal I linearized pAd/CMV-LacZ and 2.6 µl of PacI  
linearized pWE-Ad.AflIII-rITR plasmid DNA (both 1 µg/µl) and  
95 µl serum free Dulbecco's Modified Eagles Medium (DMEM)  
25 were mixed with 25.6 µl lipofectamine diluted in 74.4 µl  
serum free DMEM by adding the lipofectamine to the DNA mix.  
The DNA/lipofectamine mixture was left at room temperature  
for 30 minutes after which 1.3 ml serum free media was added.  
The latter mixture was then added (30 µl per well) to PER.C6  
30 seeded wells that were washed with 200 µl DMEM prior to  
transfection. After 3 hours in a humidified CO<sub>2</sub> incubator  
(37°C, 10% CO<sub>2</sub>) 200 µl DMEM with 10% fetal calf serum 10 mM  
MgCl<sub>2</sub> was added to each well and the plates were returned to  
the humidified CO<sub>2</sub> incubator (37°C, 10% CO<sub>2</sub>). The next day  
35 the medium of each well was replaced with 200 µl DMEM, 10%  
FCS, 10 mM MgCl<sub>2</sub>. The plates were then left in the  
humidified CO<sub>2</sub> incubator for an additional three days after

which the wells were subjected to freezing at  $-20^{\circ}\text{C}$  for at least 1 hour followed by thawing and resuspension by repeated pipetting. Transfection efficiency was determined using lacZ staining in additional plates and found to be approximately 40% for each transfected well of PER.C6 cells. An aliquot of 100  $\mu\text{l}$  of freeze/thawed transfected cells was transferred to each well of a plate with new PER.C6 cells seeded as described above without PLL coated plates (plate 2). The second 96-well plate with PER.C6 cells incubated with freeze/thaw cell lysate of the first transfected plate was checked for CPE. At least 5% of the wells showed clear CPE after 2 days. Four days after infection with the lysate from plate 1 the plate was subjected to one freeze-thaw cycle and 10  $\mu\text{l}$  from each lysed well was added to wells of a plate seeded with A549 cells ( $1 \times 10^4$  cells per well seeded in 100  $\mu\text{l}$  in DMEM, 10% FCS the day before). Two days after infection the wells were stained for lacZ activity. Of the infected wells 96% were infected and stained blue. All wells stained and a large number of wells showed 100% blue staining and thus transduction of all cells with adenoviral vector carrying lacZ. Extrapolated from MOI experiments in tissue culture flasks the adenoviral titer of well-produced virus is around  $10^6$ - $10^7$  infectious units per ml.

The subject invention discloses methods and compositions for the high throughput delivery and expression in a host of sample nucleic acid(s) encoding product(s) of unknown function. Methods are described for the construction of complementing cell lines, libraries of adenovirus derived plasmids containing sample nucleic acids, packaging the adenovirus-derived plasmids into adenovirus vectors, infecting a host with the adenovirus vectors that express the product(s) of the sample nucleic acid(s) in the host, identifying an altered phenotype induced in the host by the product(s) of the sample nucleic acids, and thereby assigning a function to the product(s) encoded by the sample nucleic



acids. The sample nucleic acids can be, for example, synthetic oligonucleotides, DNAs, cDNAs and can encode, for example, polypeptides, antisense nucleic acids, or GSEs. The methods can be fully automated and performed in a multiwell  
5 format to allow for convenient high throughput analysis sample nucleic acid libraries.

#### Example 10

10 Miniaturized, multiwell production of E1 and E2A deleted recombinant adenoviral vectors carrying therapeutic and marker transgenes

To allow the construction of cDNA libraries with a  
15 representative repertoire of cDNA sequences, the cloning capacity of the miniaturized adenoviral production system a derivative of PER.C6, namely PER.C6/E2A, was used. This cell line allows the production of a vector with three deletions of adenoviral expression cassettes: E1, E2A and E3. These  
20 three deletions allow the theoretical cloning of vectors with transgene sizes of up to about 10.5 kb in length. Here we show the production of E1 and E2A deleted vectors carrying a variety of human and mouse cDNAs as well as additional marker genes.

25 The day before transfection, PER.C6/E2A cells were harvested using trypsin-EDTA and counted. The cells were then diluted with culture medium (DMEM with 10% fetal bovine serum and 10 mM MgCl<sub>2</sub>) to a suspension of 22.500 cells per 100 µl followed by seeding 100 µl per well of poly-L-lysine (PLL) coated 96-  
30 well plates (Becton and Dickinson). The next day 2.6 µg of the linearized adapter molecules and 2.6 µg of PacI linearized pWE/Ad.AflIII-rITR.deltaE2A plasmid DNA (see example 19) in a volume of 100 µl serum free Dulbecco's Modified Eagles Medium (DMEM) were mixed with 25.6 µl  
35 lipofectamine diluted in 74.4 µl serum free DMEM by adding the lipofectamine mix to the DNA mix. The DNA/lipofectamine

mixture was left at room temperature for 30 minutes after which 1.3 ml serum free medium was added. The latter mixture was then added (30  $\mu$ l per well) to PER.C6/E2A seeded wells that were washed with 200  $\mu$ l DMEM prior to transfection.

5 After 3 hours in a humidified CO<sub>2</sub> incubator (39°C, 10% CO<sub>2</sub>) 200  $\mu$ l DMEM with 10% fetal bovine serum and 10 mM MgCl<sub>2</sub> was added to each well and the plates were returned to the humidified CO<sub>2</sub> incubator (39°C, 10% CO<sub>2</sub>). The next day the medium of each well was replaced with 200  $\mu$ l DMEM with 10%

10 fetal bovine serum and 10 mM MgCl<sub>2</sub>. The plates were then returned to a humidified CO<sub>2</sub> incubator (32°C, 10% CO<sub>2</sub>) for an additional seven days after which the wells were subjected to freezing at -20°C overnight followed by thawing and resuspension by repeated pipetting. An aliquot of 100  $\mu$ l of

15 the freeze/thawed transfected cells was transferred to each well of a plate with fresh PER.C6/E2A cells seeded as described above on normal 96-well-tissue culture plates (plate 2). The second 96-well plate with PER.C6/E2A cells incubated and thus infected with freeze/thawed cell lysate

20 of the first transfected plate was checked for CPE formation (see figure 27) and stored at -20°C. In figure 27 the percentage of virus producing cells (CPE positive) wells scored after propagation of the freeze/thawed transfected cells to new PER.C6/E2A cells is depicted. Clearly the

25 miniaturized system subject of this application allows the efficient production of deltaE1/E2A double deleted vectors with a variety of transgene inserts.

## Example 11.

Quantification of adenoviral vector particles produced in miniaturized production system using PER.C6/E2A

5

Adenoviral plaque assays were performed in order to determine the titer of the adenoviral vectors produced in one well of a 96-well-tissue-culture plate.

10 PER.C6/E2A cells were harvested using trypsin-EDTA and counted. The cells were then diluted with culture medium (DMEM with 10% fetal bovine serum and 10 mM  $MgCl_2$ ) to a suspension of  $1.5 \times 10^6$  cells per 2 ml, followed by seeding 2 ml per well of PLL coated 6-well plates (Becton and  
15 Dickinson). Microtiter plates containing adenoviral vector lysates were thawed and 50  $\mu$ l of a randomly chosen well of each adenovirus was used to make serial 10-fold dilutions of the adenovirus in culture medium. The medium of the PER.C6/E2A cells, that were seeded the same day, was replaced  
20 with 2 ml per well diluted virus and the 50-60% monolayer was infected for approximately 16 hours in a humidified  $CO_2$  incubator (32°C, 10%  $CO_2$ ). After infection the cells were overlayed with 3 ml per well agarose mix (2xMEM, 2% fetal bovine serum, 1 mM  $MgCl_2$ , PBS and 1% agarose) and returned to  
25 the humidified  $CO_2$  incubator (32°C, 10%  $CO_2$ ). After two weeks nine individual plaques, including one negative control, were transferred to 200  $\mu$ l of culture medium and stored at -20°C. An aliquot of 25  $\mu$ l of this material was used to infect  
30 PER.C6/E2A cells ( $2.25 \times 10^4$  cells per well in 100  $\mu$ l), seeded in 96-well-tissue-culture-plates one day prior to infections. This was incubated in the humidified  $CO_2$  incubator (32°C, 10%  $CO_2$ ) until the presence of full CPE was observed, and subsequently stored at -20°C.

35 The final titer of the adenoviruses, produced in a well of a 96-well-tissue-culture plate, was determined one week after

picking the individual plaques In figure 28 the titer of adenoviruses in pfu/ml, produced in a well of a 96 well plate is depicted. Average titers of  $0.8 \pm 0.7 \times 10^9$  pfu/ml imply that depending on the MOI needed in a particular cell based assay in a functional genomics screen using 384 well plates, sufficient virus is produced for 400-4000 assays (MOIs of 100-10). This allows multiple screens using one library.

10

## Example 12

The quality of adenoviral vector produced in a mikrotiter plate on PER.C6/E2A cells

15

To test for functionality of the produced recombinant adenovirus the following functional assays were performed on cells infected with the respective adenoviral vectors:

- 20 -  $\beta$ -Galactosidase assay
- hIL3 assay
- luciferase assay
- ceNOS assay
- GLVR2 assay
- 25 - EGFP assay

 $\beta$ -Galactosidase assay

- 30 A549 cells were harvested using trypsin-EDTA and counted. The cells were then diluted with culture medium (DMEM with 10% heat-inactivated FBS) to a suspension of 10.000 cells per 100  $\mu$ l, followed by seeding 100  $\mu$ l per well of 96-well-tissue-culture plates. The next day, all CPE-positive PER.C6/E2A wells containing lacZ-transducing adenoviruses as well as
- 35 negative controls (both primary wells and plaques amplified

on fresh PER.C6/E2A cells) were used to infect the A549 cells. For this purpose the frozen wells were thawed and 20  $\mu$ l of each well of the freeze/thawed cell lysate was used to infect one well of the A549 cells. Two days after infection, the medium of the infected A549 cells was removed and each well was washed two times with 100  $\mu$ l PBS (phosphate-buffered saline). After washing, the cells were fixated for five minutes at room temperature by adding 100  $\mu$ l fixative (1% formaldehyde, 0.2% glutardialdehyde) per well. After washing the cells two times with PBS, 100  $\mu$ l X-gal staining solution (0.2 M  $K_3Fe(CN)_6$ , 0.2 M  $K_4Fe(CN)_6$ , X-gal in DMSO and 0.1 M  $MgCl_2$ ) was added to each well.

All of the wells that were infected with CPE-positive wells stained blue. A large number of wells showed 100% blue staining and thus transduction of all cells with adenoviral vector carrying lacZ (see figure 29).

#### hIL-3 assay

The day before infection the A549 cells were seeded as described above. The next day, all CPE-positive PER.C6/E2A wells containing human interleukin-3 (hIL-3) transducing adenoviruses (both primary wells and plaques amplified on fresh PER.C6/E2A cells), as well as positive and negative controls, (were used to infect the A549 cells. For this purpose the frozen wells were thawed and 20  $\mu$ l of each well of the freeze/thawed cell lysate was used to infect one well of the A549 cells. Three days after infection, the quantity of hIL-3 concentrations in 100  $\mu$ l of the supernatants of the infected A549 cells was determined using the human IL-3 immunoassay (Quantikine<sup>TM</sup>).

All of the wells that were infected with CPE-positive wells showed high hIL-3 concentrations (see figure 29).

### Luciferase assay

The day before infection the A549 cells were seeded as described above. The next day, all CPE-positive PER.C6/E2A wells containing luciferase transducing adenoviruses (both primary wells and plaques amplified on fresh PER.C6E2A cells), as well as positive and negative controls, were used to infect the A549 cells. For this purpose the frozen wells were thawed and 20 µl of each well of the freeze/thawed cell lysate was used to infect one well of the A549 cells. Two days after infection, the medium of the infected A549 cells was removed and each well was washed with 100 µl PBS. After adding 100 µl 1x reporter lysis buffer (Promega) the wells were subjected to freeze/thawing, followed by measuring the luciferase activity in 20 µl of the freeze/thawed cell lysates.

All of the wells that were infected with CPE-positive wells showed a high luciferase activity (see figure 29).

### ceNOS- assay

PER.C6/E2A cells were harvested using trypsin-EDTA and counted. The cells were then diluted with culture medium (DMEM devoid of phenol-red with 10% FBS and 10 mM MgCl<sub>2</sub>) to a suspension of 22.500 cells per 100 µl, followed by seeding 100 µl per well of 96-well-tissue-culture plates. The next day, all CPE-positive PER.C6/E2A wells containing ceNOS transducing adenoviruses (both primary wells and plaques amplified on fresh PER.C6/E2A cells), as well as positive and negative controls, were used to infect the PER.C6/E2A cells. For this purpose the frozen wells were thawed and 20 µl of each well of the freeze/thawed cell lysate was used to infect one well of the PER.C6/E2A cells. Three days after infection, 50 µl color solution [GreissA reagent (0.1% N-(1-

Naphtyl)Ethylenediamine) and GreissB reagent (25% Sulfanylamide in 5% phosphoric acid) in a 1:1 ratio] was added to 50 µl of the supernatants of the infected PER.C6E2A cells. After adding the color solution, supernatants with a positive ceNOS activity turned directly pink.

All of the wells that were infected with CPE-positive wells showed a positive ceNOS activity (see figure 29).

#### 10 GLVR2 amphotropic receptor assay

Adenovirus mediated transduction of GLVR2 the receptor for amphotropic retroviruses was measured essentially as described (Lieber et al, 1995), except for the use of an amphotropic retroviral supernatant transferring a truncated version of the human nerve growth receptor (NGFR). Retroviral transduction of the CHO cells infected with GLVR2 adenoviral supernatant was detected using anti-NGFR antibodies and a flow cytometer.

All of the wells that were infected with CPE-positive PER.C6/E2A wells containing GLVR2 transducing adenoviruses (plaques amplified on fresh PER.C6/E2A cells) showed a positive GLVR2 activity (see figure 29).

#### 25 EGFP assay

EGFP expression was measured on a microtiter plate fluorimeter or by flow cytometer.

In conclusion both virus produced from wells as well as virus plaque purified (i.e. cloned) from producing wells showed transduction of their respective transgenes. Therefore the system shows high fidelity for the production of functional adenoviral vectors and produces no aberrant forms for the transgene inserts tested.

## Example 13

DNA isolation methods generating  
sufficiently purified plasmid DNA  
5 for production of adenoviral vectors in PER.C6 and  
PER.C6-E2A cells

It is well known that plasmid DNA that is used for  
10 transfection studies in eukaryotic cells must be of  
sufficient purity and free of endotoxins to achieve high  
levels of transfection efficiencies. Conventional methods for  
purifying plasmid DNA from E. coli include an alkaline lysis  
procedure (Birnboim, H.C. and Doly, J, (1979) A rapid  
15 alkaline lysis procedure for screening recombinant plasmid  
DNA. Nucleic Acid Res. 7: 1513-1522) followed by either  
banding of the plasmid DNA on caesium chloride (CsCl)  
gradients (see Sambrook, J. et al, eds. (1989) Molecular  
cloning: a laboratory manual, 2<sup>nd</sup> edition, Cold Spring Harbor  
20 Laboratory Press), or by binding and elution on an anion-  
exchange resin (see, for example, Qiagen<sup>TM</sup> plasmid  
purification methods of Qiagen Inc.; and Concert<sup>TM</sup> plasmid  
purification systems of Life Technologies). However, all of  
these methods are unsuited for high throughput DNA  
25 isolations, since they require considerable hands-on time per  
isolation. Therefore, and to reduce the costs per isolation,  
other methods were examined.

Methods that were examined using the SalI linearized  
30 adenoviral adapter plasmid pCLIP-SalI LacZ and the E2A  
deleted helper fragment pWE/Ad.AflIII-rITR.deltaE2A:  
alkaline lysis followed by column based plasmid purification  
(Qiagen)

1. alkaline lysis followed by isopropanol precipitation,  
35 and solubilization in TE buffer



2. alkaline lysis followed by isopropanol precipitation,  
and solubilization in TE buffer containing RNase at  
10 microgram per ml
3. alkaline lysis followed by isopropanol precipitation,  
5 and solubilization in TE buffer containing RNase at  
10 microgram per ml, followed by phenol/chloroform  
extraction and ethanol precipitation
4. Standard cetyltrimethylammonium bromide (CTAB)  
plasmid isolation (Nucleic Acids Res, 16<sup>20</sup>;1488)
- 10 5. Standard CTAB plasmid isolation, but solubilization  
in TE buffer containing RNase at 10 microgram per ml,  
followed by phenol/chloroform extraction

Equal volumes of the resulting plasmids were linearized with  
15 SalI, followed by phenol/chloroform extraction and ethanol  
precipitation. Following solubilization in TE buffer and  
checking on an agarose gel, equal amounts of DNA (as  
determined by the ethidium bromide staining) were transfected  
into PER.C6/E2A cells with lipofectamine as described under  
20 examples 9 and 10. After propagation, wells were scored for  
CPE formation, as a measure for virus production.

In figure 30 the relative amounts of wells producing  
adenoviral vector (CPE positive) after transfection of  
25 PER.C6/E2A cells transfected with pCLIP-LacZ, purified by 6  
different protocols. Qiagen: standard alkaline lysis followed  
by Qiagen plasmid purification; AlkLys: alkaline lysis  
followed by isopropanol precipitation, and solubilization in  
TE buffer; AL + RNase: alkaline lysis followed by isopropanol  
30 precipitation, and solubilization in TE buffer containing  
RNase at 10 microgram per ml; AL+R+phenol: alkaline lysis  
followed by isopropanol precipitation, and solubilization in  
TE buffer containing RNase at 10 microgram per ml, followed  
by phenol/chloroform extraction and ethanol precipitation;  
35 CTAB: Standard CTAB plasmid isolation CTAB+phenol: Standard  
CTAB plasmid isolation, but solubilization in TE buffer

containing RNase at 10 microgram per ml, followed by phenol/chloroform; It is evident that the quality of the DNA is not a major determinant for transfection of, and virus production in, PER.C6/E2A cells, as all 6 differently  
5 isolated plasmids produced similar amounts of wells with CPE.

In conclusion: for high throughput transfection of, and virus production in, PER.C6/E2A cells, it is sufficient to use plasmid DNA that was precipitated with 0.6 volumes of  
10 isopropanol after standard alkaline lysis, followed by solubilization in TE buffer.

#### Example 14

15 The use of unpurified, digested adapter and helper adenoviral DNA molecules for the generation of adenoviral vectors in a miniaturized format

20 In order to minimize the overall costs, and the chances for errors in the procedure, plus to maximize the throughput when producing recombinant adenoviruses in a high throughput fashion, it is desirable to leave out as many steps as possible. Any improvement here is also applicable when  
25 generating adenoviral vectors on a smaller, low throughput scale. The most difficult step to automate when producing recombinant adenoviruses is a DNA clean-up step by phenol chloroform extraction(p/c) prior to transfection of cells with the DNAs. DNA is purified after linearization in order  
30 to obtain enzyme-free DNA. This is thought to be important to obtain high percentages of virus generation after transfection with the adapter and helper DNA molecules. An additional motivation to leave out the p/c purification procedure, is the risk of traces of phenol and chloroform in  
35 the DNA used for transfection, which can have a negative

effect on the generation of viruses. Therefore it was investigated whether the complicated step of p/c clean-up could be omitted from the miniaturized adenoviral vector generation protocol subject of this application. This method  
5 forms the basis of high throughput construction of libraries, such as sense or antisense cDNA expression libraries. Several independent experiments were performed in order to test the effect of omitting the p/c step on the efficiency of adenoviral vector generation. The p/c purification was  
10 carried out as follows. After digesting the adapter-DNA and rITR-DNA with the appropriate restriction enzymes, an equal volume of phenol and chloroform (1:1) was added, mixed thoroughly and centrifuged (5 minutes, 14,000 rpm). The aqueous phase was transferred to a new micro-centrifuge tube  
15 and an equal volume of chloroform was added. Again, this was mixed thoroughly and centrifuged (5 minutes, 14,000 rpm). The aqueous phase was transferred to a new microcentrifuge tube and one-tenth volume 3M sodium acetate (pH 5.2) and 2.5 volumes absolute ethanol were added. This was kept at -20°C  
20 for at least 20 minutes, subsequently centrifuged (15 minutes, 14,000 rpm) and the pellet was washed with 70% ethanol. The DNA was air-dried and a suitable volume of sterile water was added (in Laminar Airflow Cabinet). Transfection was carried out as described in examples 9 and  
25 beyond using PER.C6/E2A cells. All viruses are E1 and E2A deleted and were produced in PER.C6/E2A cells.

In the first experiment, adapter-DNA containing  $\beta$ -galactosidase (pAd5.Clipsal.LacZ) of 6 different DNA  
30 isolation protocols (as described in example 13) were analyzed and compared for their efficiency in producing adenoviral vector by monitoring for CPE formation. Of all DNA samples, half was p/c purified after linearization using the appropriate restriction enzyme (SalI), and half was not

purified after linearization. The restriction enzyme was heat inactivated because a SalI site is present in the rITR delta E2A helper fragment that was used and thus to exclude inadvertant digestion of the helper DNA. In this experiment  
5 p/c purified rITR delta E2A helper DNA was used was used. In all of the used DNA isolation methods, CPE was formed efficiently (figure 31A). I some cases ommittance of purification by phenol chloroform extarction gave higher CPE efficienciencies. In conclusion adenoviral adapter DNA digested  
10 with the linearizing enzyme can be used for transfection without prior purification.

In the second experiment, purifying and not purifying were compared using adapter-DNA containing Enhanced Green  
15 Fluorescent Protein (EGFP) and Enhanced Yellow Fluorescent Protein (EYFP) (pAd5.Clippac.EGFP and pAd5.Clippac.EYFP). The adapter plasmid-DNA was isolated using the Qiagen isolation method. The rITR delta E2A used was p/c purified. The adapter-DNA was linearized using PacI, which did not have to  
20 be heat inactivated before transfection because there is no PacI site present in the rITR. No consistent differences were found in the percentages of CPE observed and production of adenoviral vector was efficient (figure 31 B).

The third experiment contained an extra variable. The need to  
25 purify the rITR was tested. The used adapter DNA contained EGFP (pAd5.Clippac.EGFP) and was isolated, using the Qiagen isolation method (figure 30C). The results after transfection and propagation show that the purification of both adapter and rITR DNA after restriction enzyme digestion is not  
30 necessary.

Taking all results in account, it is clear that the phenol chloroform purification step of the adapter DNA and rITR is not obligatory to obtain high percentages of CPE, and thus

for adenoviral vector production. The above described modification of the procedure as for example described in examples 9 and 10 results in a significant increase in throughput when generating adenoviral vector libraries in an automated setup, and when making vectors manually on a smaller scale.

#### **Example 15**

Production of adenoviral vectors in relation to stability of the produced vector.

Generation of recombinant adenoviruses as described in the various examples herein indicates that a functional adenovirus will be formed approximate five to eleven days after amplification of the virus produced on the transfected PER.C6 cells (and derivatives) grown in multiwell tissue culture plates. The observation of a cytopathic effect (CPE) indicates that functional adenovirus has been formed and is replicating. The nature of the transgene inserts and variations in the experimental conditions cause the kinetics of virus generation to be variable. In a high throughput setting where large numbers of wells and thus plates containing adenoviral vector are handled, it is desirable to have a single point in time to harvest the plates and score for adenoviral vector production. The above mentioned variations in adenoviral vector generation may be overcome by postponing the harvest of the plates as long as possible i.e. until the slower wells also have produced adenoviral vector. For this purpose we tested the stability of recombinant lacZ adenovirus (pCLIP-lacZ) once it is produced starting from low numbers of virus to higher numbers of virus and then the titers were determined (see example 11) plus lacZ transduction-potential of the virus after up to three weeks.

PER.C6/E2A cells were seeded in 2 rows of 96-well microtiter tissue culture plates using  $4 \cdot 10^4$  cells/well. The plates were incubated overnight at 39 °C. The next day PER.C6/E2A was infected with purified LacZ-adenoviral vector of serotype 5.

5 The infections were done at different MOIs according to the scheme below (21 plates in total).

Table 1

10

	1	2	3	4	5	6	7	8	9	10	11	12
<u>A</u>												
<u>B</u>	0.01	0.01	0.01	0.1	0.1	0.1	1	1	1	10	10	10
<u>C</u>	0	0	0	0	0	0	0	0	0	0	0	0

To determine the effect of temperature on stability of adenoviruses produced in wells, seven plates were incubated at 32°C, seven plates at 34°C and seven plates at 39°C.

15 At day 2; 3; 6; 9; 13; 16 and 21 after infection, one plate corresponding to each incubation temperature was frozen. The cell lysates were used in the following experiments.

In order to determine the transduction potential of the produced adenoviruses, A549 cells were seeded in 96-well

20 microtiter tissue culture plates with  $1 \cdot 10^4$  cells/well and incubated overnight at 37°C. Then, the cells were infected with 50 µl cell lysate and incubated at 37°C. After two days the cells were screened for toxicity followed by lacZ staining. A clear toxic effect was observed with increasing

25 MOI and increasing time of infection. The table below is a summary of when all cells stained blue in all wells.

Table 2

	moi	# days after infection.
32°C	0.001	9
	0.01	9
	0.1	6
	1	6
34°C	0.001	6
	0.01	6
	0.1	6
	1	6
37°C	0.001	6
	0.01	6
	0.1	3
	1	3

Three weeks after infection 100% blue cells/well are still  
 5 observed and thus all cells with adenoviral vector carrying  
 lacZ. Thus this showed no decreased infectivity upon  
 incubation up to 3 weeks.

In order to determine the number of infectious virus  
 particles of virus in the cell lysate a titration assay was  
 10 performed for the samples which were incubated 2, 6 and 21  
 days after infection corresponding to each incubation  
 temperature and MOI. Three weeks after infection an average  
 titer of  $2 \times 10^{10}$  pfus per ml was observed. An overview of  
 the titers is given in figure 32.

15

The above mentioned experiments indicate that variations in  
 adenoviral vector generation may be overcome by postponing  
 the harvest of the plates as long as possible i.e. until the  
 slower wells also have produced adenoviral vector. Although  
 20 we see a clear toxic effect with increasing MOI and  
 increasing time of infection, there is no decreased

infectivity and no decrease in titer of the produced adenoviral vector.

#### Example 16

5

Miniaturized production of adenoviral vectors carrying antisense DNA sequences and expressing antisense mRNA sequences.

10

Decreasing endogenous gene expression in screens using antisense cDNA expression libraries are very useful in functional genomics programs. Individual antisense adenoviral vectors can also be used for gene validation and the development of an antisense gene therapeutic. An example is the use of antisense-Vascular Endothelial Growth Factor (VEGF). VEGF is a pivotal molecule in tumoral angiogenesis that promotes endothelial cell growth and plays a major role in neovascularization and growth of gliomas. The VEGF-antisense molecule inhibits tumor growth *in vivo*. (Seock-Ah et al., 1999, Cancer research 59, 895-900). Constructing large and complex antisense libraries in adenoviral vectors are a valuable and very useful for Functional Genomics screening programs.

25

PER.C6/E2A cells were cotransfected with linearized adapter DNA, containing a defined human cDNA sequence in antisense orientation, and linearized rITR delta E2A helper DNA, as described in example 10. The genes cloned in antisense orientation in adapter DNA are described in table below. For pCLIP, two variants were used with SalI or PacI as the site to linearize depending on the transgene inserts.

30



Table 3

Antisense-cDNA	Abbreviation	Adenoviral vector
constitutive nitric oxide synthase	CeNOS	pCLIP, pIPspAdapt
lysosomal beta-glucocerebrosidase	hGC	pCLIP
Phenol UDP-glucuronosyltransferase	P-UGT	pCLIP, pIPspAdapt
Bilirubin 1 UDP-glucuronosyltransferase	B-UGT	pCLIP, pIPspAdapt
Plasminogen Activator Inhibitor type-1	PAI-1	pCLIP, pIPspAdapt
Ribosomal protein L4	NA	pCLIP
Phosphoenolpyruvate carboxykinase	PEPCK	pCLIP
$\beta$ -globin	NA	pCLIP
Lysozyme	NA	pCLIP
Chrom 1 spec. transcr. KIAA0493	KIAA	pCLIP
snRNP core protein Sm D2	SnRNP	pCLIP

In **figure 33** an example is given of some of the above  
5 mentioned cDNAs for generation of antisense cDNA adenoviral  
vectors using the miniaturized production system subject of  
this invention. These viruses will be used to attempt to  
lower the endogenous expression of the cells tested.

## Example 17

Construction of adapter plasmids for the generation and production of recombinant adenoviruses, in particular, for the generation and production of adenoviral expression libraries.

Adenoviral adapter plasmids (in short adapter) were constructed that contain multiple cloning sites in multiple orientations that allow efficient cloning of sense or anti-sense cDNA sequences and the generation of libraries of nucleic acids including cDNA libraries in these vectors. Furthermore, these new adapter plasmids contain novel restriction enzyme recognition sequences bordering the left adenoviral ITR and the sequences overlapping with the helper fragment until nucleotide 6095 of the Ad5 viral genome. These modifications of the adenoviral adapter plasmids significantly enhance the possibility to linearize the adapter plasmids, without digestions of inserted transgenes or transgene libraries. Following cotransfection with pWE/Ad.AflIII-rITR.deltaE2A, homologous recombination between the improved adapter plasmids and the adenoviral cosmid results in the generation of functional adenoviruses.

25

The first adapter constructs, pCLIP-IppoI (figure 34A) and pCLIP-IppoI-polynew (figure 34B) are derived from pAd5/CLIP-Pac and contain a new I-PpoI linearization site at position -11 bp in front of the left ITR. In addition, pCLIP-IppoI-polynew contains an improved poly-linker sequence downstream of the CMV promoter encompassing restriction enzyme recognition sequences for different rare cutting restriction endonucleases and intron-encoded

30

endonucleases. The recognition sequences for intron-encoded endonucleases are extremely rare in genomes including the human genome, and consist of 11-23 base pairs. As these intron-encoded endonuclease-sites are absent in the adenoviral genome, sequences can directly be inserted into a full adenoviral vector genome obtained from an insertless pCLIP-IPpoI (see also below).

To construct this adapter plasmid, part of the left ITR of Ad5 was amplified by PCR on pCLIP-PacI template plasmid DNA using the following primers: PCLIPPACIPPO: 5'- TTT TTA ATT AAT AAC TAT GAC TCT CTT AAG GTA GCC AAA TCA TCA TCA ATA ATA TAC CTT ATT TTG G- 3' and PCLIPBSRGI: 5'- GCG AAA ATT GTC ACT TCC TGT G - 3' and Elongase polymerase from Life Technologies (LTI; Breda, The Netherlands). Primer pCLIP-PacI contains a PacI site 5' from a I-PpoI sequence. The amplified fragment was digested with *PacI* and *BsrGI* and the resulting fragment of 255 bp cloned into a fragment of 6471 bp which was obtained from pAd5/CLIP-PacI digested with the same enzymes and isolated on a 1 % agarose gel. Nucleotide sequences were confirmed by dideoxynucleotide sequence analysis. This construct, containing *PacI* and *I-PpoI* recognition sequences 5' to the left ITR at a distance of 33 nucleotides and 11 nucleotides, respectively, was named pCLIP-I-PpoI (see figure 34A) This construct was

subsequently digested with *XbaI* and *HindIII* separated on a gel and used to insert a new synthetic linker sequence. This linker sequence, composed of the two single stranded and annealed oligonucleotides: LINKERPOLYNEW-S: 5'-AGC TTT AAC TAT AAC GGT CCT AAG GTA GCG ATT AAT TAA CAG TTT AAT TAA TGG CAA ACA GCT ATT ATG GGT ATT ATG GGT T- 3'; and LINKERPOLYNEW-AS: 5'-CTA GAA CCC ATA ATA CCC ATA ATA GCT GTT TGC CAT TAA TTA AAC TGT TAA TTA ATC GCT ACC TTA GGA CCG TTA TAG TTA A- 3' was directly ligated into the digested construct. This adapter construct, termed pCLIP-I-PpoI-

polynew, now contains recognition sequences for the restriction enzymes *HindIII*, *I-CeuI*, *PacI*, *Pi-PspI* and *XbaI* in the polylinker (see figure 34B). Correct insertion of this linker was verified by digestions with the respective enzymes and sequence analysis.

A different adapter construct, pADAPT, which contains a stronger CMV promoter than pCLIP-based adenoviral adapters as well as a different poly(A) sequence, was used as a backbone to construct another set of adapter plasmids. To enhance the linearization possibilities a number of pADAPT derivatives were designed and constructed. For this purpose pADAPT plasmid DNA was digested with *SalI* and treated with Shrimp Alkaline Phosphatase to reduce religation. A linker, composed of the following two phosphorylated and annealed oligo's: ExSalPacF 5'-TCG ATG GCA AAC AGC TAT TAT GGG TAT TAT GGG TTC GAA TTA ATT AA- 3'; and ExSalPacR 5'-TCG ATT AAT TAA TTC GAA CCC ATA ATA CCC ATA ATA GCT GTT TGC CA- 3'; was directly ligated into the digested construct, thereby replacing the *SalI* restriction site by *Pi-PspI*, *SwaI* and *PacI*. Furthermore, part of the left ITR of pADAPT was amplified by PCR using the following primers: PCLIPMSF: 5'-CCC CAA TTG GTC GAC CAT CAT CAA TAA TAT ACC TTA TTT TGG -3' and pCLIPBSRGI (see above). The amplified fragment was digested with *MunI* and *BsrGI* and cloned into pCLIP-EcoRI, which was partially digested with *EcoRI* and after purification digested with *BsrGI*. After restriction enzyme analysis, the construct was digested with *ScaI* and *SgrAI* and an 800 bp fragment was isolated from gel and ligated into *ScaI/SgrAI* digested pADAPT+ExSalPac linker. The resulting construct, named pIPspSalAdapt (**see figure 34C**), was digested with *SalI*, dephosphorylated, and ligated to the abovementioned phosphorylated ExSalPacF/ExSalPacR

doublestranded linker. A clone in which the *PacI* site was closest to the ITR was identified by restriction analysis and sequences were confirmed by sequence analysis. This novel pADAPT construct, termed pIPspAdapt (**see figure 34D**) thus harbours two ExSalPac linkers containing recognition sequences for *PacI*, *PI-PspI* and *BstBI*, which surround the adenoviral part of the adenoviral adapter construct, and which can be used to linearize the plasmid DNA prior to cotransfection with adenoviral helper fragments.

In order to further increase transgene cloning permutations a number of polylinker variants were constructed based on pIPspAdapt. For this purpose pIPspAdapt was first digested with *EcoRI* and dephosphorylated. A linker composed of the following two phosphorylated and annealed oligo's:

Ecolinker<sup>+</sup>: 5'-AAT TCG GCG CGC CGT CGA CGA TAT CGA TAG CGG CCG C 3' and Ecolinker<sup>-</sup>: 5'-AAT TGC GGC CGC TAT CGA TAT CGT CGA CGG CGC GCC G 3' was ligated into this construct,

thereby creating restriction sites for *AscI*, *SallI*, *EcoRV*, *ClaI* and *NotI*. Both orientations of this linker were obtained and sequences were confirmed by restriction analysis and sequence analysis. The plasmid containing the polylinker in the order 5' *HindIII*, *KpnI*, *AgeI*, *EcoRI*, *AscI*, *SallI*, *EcoRV*, *ClaI*, *NotI*, *NheI*, *HpaI*, *BamHI* and *XbaI* was termed pIPspAdapt1 (**see figure 34E**) while the plasmid containing the polylinker in the order *HindIII*, *KpnI*, *AgeI*, *NotI*, *ClaI*, *EcoRV*, *SallI*, *AscI*, *EcoRI*, *NheI*, *HpaI*, *BamHI* and *XbaI* was termed pIPspAdapt2 (**see figure 34F**).

Those skilled in the art of making cDNA libraries will appreciate that an extra polylinker, consisting of the oligos GalMlu-F: 5'-CGA TCG GAC CGA CGC GTT CGC GAG C-3' and GalMlu-R: 5'-GGC CGC TCG CGA ACG CGT CGG TCC GAT-3', was inserted in between the *ClaI* and *NotI* sites of

pIPspAdapt1, to generate pIPspAdapt6 (**see figure 34G**).

pIPspAdapt6 contains extra restriction sites for *RsrII*,

*MluI* and *NruI*, which were introduced to increase the

distance between the *SalI* and *NotI* sites, which will

5 improve the digestion of the combination of these enzymes.

Furthermore, they allow the pre-digestion and

dephosphorylation of this vector prior to restriction with

*SalI* and *NotI*, which will reduce background recombinants in

the case of cloning individual inserts or libraries with

10 *SalI*- and *NotI*-compatible overhangs. The GalMlu oligo was

also cloned into pIPspAdapt2, leading to pIPspAdapt7 (**see**

**figure 34H**).

To facilitate the cloning of other sense or antisense

15 constructs, a linker composed of the following two

oligonucleotides was designed, to reverse the polylinker of

pIPspAdapt: HindXba<sup>+</sup> 5'-AGC TCT AGA GGA TCC GTT AAC GCT AGC

GAA TTC ACC GGT ACC AAG CTT A-3'; HindXba<sup>-</sup> 5'-CTA GTA AGC

TTG GTA CCG GTG AAT TCG CTA GCG TTA ACG GAT CCT CTA G-3'.

20 This linker was ligated into *HindIII/XbaI* digested

pIPspAdapt and the correct construct was isolated.

Confirmation was done by restriction enzyme analysis and

sequencing. This new construct, **pIPspAdaptA (see figure**

**34I)**, was digested with *EcoRI* and the above mentioned

25 *EcoI*inker was ligated into this construct. Both

orientations of this linker were obtained, resulting in

pIPspAdapt3 (**see figure 34J**), which contains the polylinker

in the order *XbaI*, *BamHI*, *HpaI*, *NheI*, *EcoRI*, *AscI*, *SalI*,

*EcoRV*, *ClaI*, *NotI*, *AgeI*, *KpnI* and *HindIII*. pIPspAdapt4

30 contains the polylinker in the order *XbaI*, *BamHI*, *HpaI*,

*NheI*, *NotI*, *ClaI*, *EcoRV*, *SalI*, *AscI*, *EcoRI*, *AgeI*, *KpnI* and

*HindIII* (**see figure 34K**). All sequences were confirmed by

restriction enzyme analysis and sequencing.

As mentioned above, intron-encoded endonucleases are rare-cutting enzymes and do not digest the adenoviral genome. Those skilled in the art will appreciate that these enzymes allow the direct ligation of sequences in the adenoviral genome, since they do not have a recognition sequence in the adenoviral genome. To obtain a pADAPT version that contains recognition sequences for intron-encoded endonucleases in the polylinker, a linker was ligated into *HindIII/XbaI* digested pIPspAdapt, consisting of the single stranded sequences: 5'-AGC TTA ACT ATA ACG GTC CTA AGG TAG CGA TAG GGA TAA CAG GGT AAT TAA TTA ATT TAA ATT AAT TAA TCT ATG TCG GGT GCG GAG AAA GAG GTA ACT ATG ACT CTC TTA AGG TAG CCA AAT-3'; and 5'-CTA GAT TTG GCT ACC TTA AGA GAG TCA TAG TTA CCT CTT TCT CCG CAC CCG ACA TAG ATT AAT TAA TTT AAA TTA ATT AAT TAC CCT GTT ATC CCT ATC GCT ACC TTA GGA CCG TTA TAG TTA-3'. This linker was composed of four oligo's: IntrolinkerF1, IntrolinkerF2, IntrolinkerR1 and IntrolinkerR2, and contains recognition sequences for the intron-encoded endonucleases *I-CeuI*, *I-SceI*, *PI-SceI* and *I-PpoI* and the endonucleases *PacI* and *SwaI*. The correctness of the construct was confirmed by sequence analysis and the construct was termed pIPspAdapt5 (see figure 34L).

Adenoviral DNA from viruses containing pIPspAdapt5 or pCLIP-IppoI-polynew was isolated and cloned into the cosmid vector pWE15/SnaB1. pWE15/SnaB1 was created by auto-annealing the phosphorylated oligonucleotide PacSna: 5'-TAA TAC GTA TTA AT-3' and ligating the resulting double stranded sequence in *PacI*-digested and dephosphorylated pWE15/PAC, a derivative of pWE15 (see Sambrook, J. et al, eds. (1989) Molecular cloning: a laboratory manual, 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press). This generates a restriction site for *SnaB1*, which is flanked by *PacI* sites. For the generation of adenoviral DNA-containing

cosmid, blunt-ended adenoviral DNA was isolated according to standard laboratory procedures, using DNase, Proteinase K, followed by elution on an anion-exchange resin spin column. A molar excess of the resulting purified adenoviral DNA was ligated into SnaB1-restricted pWE15/SnaB1 and the resulting ligation mixture was transfected into *E. coli* Stb12 cells (LTI, Breda).

The resulting plasmid DNA was subsequently used for in vitro ligations (**see figure 34M**). The use of pIPspAdapt5-derived cosmid DNA will be used as an example in the following: Double stranded oligonucleotides containing compatible overhangs were ligated between the I-CeuI and PI-SceI sites, between I-CeuI and I-PpoI, between I-SceI and PI-SceI, and between I-SceI and I-PpoI. The PacI restriction endonuclease was subsequently used not only to linearize the construct after ligation and thereby to liberate the left- and right ITR, but also to eliminate non-recombinants. In this case, ligation mixtures can directly be used for transfection in PER.C6/PER.C6/E2A packaging cells or variants thereof, thereby eliminating the need for a cross-over or homologous recombination event to generate functional adenovirus.

As an alternative, adapter plasmids and cosmids containing adenoviral DNA made from pIPspAdapt5 or pCLIP-IppoI-polynew were used to generate fragments either encompassing the region between the left ITR and the first part of the polylinker, or encompassing the second part of the polylinker until the right ITR. Care is taken that the left and right ITR are linearized with distinct and non-compatible restriction enzymes, since ligation efficiencies are strongly reduced otherwise. pIPspAdapt5-derived cosmid DNA will be used as an example:

Plasmid pIPspAdapt5 was cut with either BstBI and I-CeuI or BstBI and I-SceI to generate the adenoviral fragment



containing the left ITR. The cosmid containing the pIPspAdapt5-derived adenoviral DNA was restricted with I-PpoI and PacI or PI-SceI and PacI to generate the fragment containing the right ITR. Fragments containing the left and right ITR were isolated on a 0.8% agarose gel and purified using anion exchange resins. Subsequently, double stranded oligonucleotides containing compatible overhangs for either I-CeuI or I-SceI at the 5' end and I-PpoI or PI-SceI at the 3' end were ligated in equimolar amounts with the fragments containing the left and right ITR's. The resulting ligation mixture was used for transfection into PER.C6/PER.C6/E2A packaging cells or variants thereof, again eliminating the need for a cross-over or homologous recombination event to generate functional adenovirus.

The direct transfection of *in vitro* ligated products benefits from an alternative way to isolate the adenoviral vector DNA. To improve the efficiency of virus production after transfection of *in vitro* ligation reactions, adenoviral vector DNA can be isolated from purified adenoviral particles (see Pronk, R. et al., Chromosoma 102: S39-S45 (1992)). This virion DNA contains 2 molecules of Terminal Protein (TP) covalently bound to the ITR sequences. It is known that TP-DNA stimulates adenovirus DNA replication over 20 fold compared to protein-free DNA. Therefore, pIPspAdapt5- or pCLIP-IppoI-polynew-derived adenoviral DNA can be isolated from virions using guanidinium hydrochloride as described (Van Bergen, B. et al. Nucleic Acids Res. 11: 1975-1989 (1983)). This DNA was digested with a suitable combination of intron-encoded restriction endonucleases and used for *in vitro* ligation reactions. After ligation, non-recombinants were removed by digestion with PacI. Further procedures were as described above and in example 10 and beyond. pIPspAdapt adapter plasmids were co-transfected with pWE/Ad.AflIII-rITRDE2A in the PER.C6/E2A packaging cells to generate recombinant

adenoviruses, as is shown in **Figure 34N** for pIPspAdapt2 as an example.

### Example 18

5

E1-deleted or E1+E2A-deleted recombinant adenoviruses with deletions in the E3 region for cloning of larger DNA inserts in miniaturized adenoviral vector production system

- 10 It is known that none of the E3-encoded proteins is required for adenoviral replication, packaging, and infection in cultured cells. This allows the possible removal of the E3 region from recombinant adenoviruses, creating opportunity for inserting large genes or complex regulatory elements
- 15 without exceeding the maximal packaging capacity. For example, part of the E3 region can be removed by deleting a *XbaI-XbaI* fragment (corresponding to Ad5 wt sequence 28592-30470). Another example is an expanded deletion of the E3 region in which sequences between the stop codon of pVIII and
- 20 the translation initiation codon of fiber (corresponding to Ad5 wt sequence 27865-30995) were removed.

Generation of pWE/Ad.AflIII-rITRAE2A:

- Deletion of the E2A coding sequences from pWE/Ad.AflIII-rITR
- 25 (ECACC deposit P97082116) has been accomplished as follows. The adenoviral sequences flanking the E2A coding region at the left and the right site were amplified from the plasmid pBr/Ad.Sal.rITR (ECACC deposit P97082119) in a PCR reaction with the Expand PCR system (Boehringer) according to the
- 30 manufacturers protocol. The following primers were used:

Right flanking sequences  
(corresponding Ad5 nucleotides 24033  
to 25180):

- ΔE2A.SnaBI: 5'-GGC GTA CGT AGC CCT GTC GAA AG-3'
- 35 ΔE2A.DBP-start: 5'-CCA ATG CAT TCG AAG TAC TTC CTT  
 CTC CTA TAG GC-3'

The amplified DNA fragment was digested with SnaBI and NsiI (NsiI site is generated in the primer  $\Delta$ E2A.DBP-start, underlined).

Left flanking sequences (corresponding Ad5 nucleotides 21557 to 22442):

$\Delta$ E2A.DBP-stop: 5'-CCA ATG CAT ACG GCG CAG ACG G-3'

$\Delta$ E2A.BamHI: 5'-GAG GTG GAT CCC ATG GAC GAG-3'

The amplified DNA was digested with BamHI and NsiI (NsiI site is generated in the primer  $\Delta$ E2A.DBP-stop, underlined).

Subsequently, the digested DNA fragments were ligated into SnaBI/BamHI digested pBr/Ad.Sal-rITR. Sequencing confirmed the exact replacement of the DBP coding region with a unique NsiI site in plasmid pBr/Ad.Sal-rITR $\Delta$ E2A. The unique NsiI site can be used to introduce an expression cassette for a gene to be transduced by the recombinant vector.

The deletion of the E2A coding sequences was performed such that the splice acceptor sites of the 100K encoding L4-gene at position 24048 in the top strand was left intact. In addition, the poly adenylation signals of the original E2A-RNA and L3-RNAs at the left hand site of the E2A coding sequences were left intact. This ensures proper expression of the L3-genes and the gene encoding the 100K L4-protein during the adenovirus life cycle.

Next, the plasmid pWE/Ad.AflIII-rITR $\Delta$ E2A was generated. The plasmid pBr/Ad.Sal-rITR $\Delta$ E2A was digested with BamHI and SpeI. The 3.9-Kb fragment in which the E2A coding region was replaced by the unique NsiI site was isolated. The pWE/Ad.AflIII-rITR was digested with BamHI and SpeI. The 35 Kb DNA fragment, from which the BamHI/SpeI fragment containing the E2A coding sequence was removed, was isolated. The fragments were ligated and packaged using  $\lambda$  phage-packaging extracts according to the manufacturer protocol (Stratagene), yielding the plasmid pWE/Ad.AflIII-rITR $\Delta$ E2A.

This cosmid clone can be used to generate adenoviral vectors that are deleted for E2A by cotransfection of PacI digested DNA together with digested adapter plasmids onto packaging

cells that express functional E2A gene product. Examples of E2A complementing cell lines are described infra and in:

5 Generation of pBr/Ad.Bam-rITRsp and pWE/Ad.AflIII-rITRsp

The 3' ITR in the vector pWE/Ad.AflIII-rITR does not include the terminal G-nucleotide. Furthermore, the PacI site is located almost 30 bp from the right ITR. Both these  
10 characteristics may decrease the efficiency of virus generation due to inefficient initiation of replication at the 3' ITR. Note that during virus generation the left ITR in the adapter plasmid is intact and enables replication of the virus DNA after homologous recombination.

15 To improve the efficiency of initiation of replication at the 3' ITR, the pWE/Ad.AflIII-rITR was modified as follows: construct pBr/Ad.Bam-rITRpac#2 was first digested with PacI and then partially digested with AvrII and the 17.8-kb vector containing fragment was isolated and dephosphorylated using  
20 SAP enzyme (Boehringer Mannheim). This fragment lacks the adenoviral sequences from nucleotide 35464 to the 3' ITR. Using DNA from pWE/Ad.AflIII-rITR as template and the primers ITR-EPH:

5'-CGG AAT TCT TAA TTA AGT TAA CAT CAT CAA TAA TAT ACC-3' and  
25 Ad101: 5'-TGA TTC ACA TCG GTC AGT GC-3'

a 630 bp PCR fragment was generated corresponding to the 3' Ad5 sequences. This PCR fragment was subsequently cloned in the vector pCR2.1 (Invitrogen) and clones containing the PCR fragment were isolated and sequenced to check correct  
30 amplification of the DNA. The PCR clone was then digested with PacI and AvrII and the 0.5 kb adeno insert was ligated to the PacI/ partial AvrII digested pBr/Ad.Bam-rITRpac#2 fragment generating pBr/Ad.Bam-rITRsp. Next this construct was used to generate a cosmid clone that has an insert  
35 corresponding to the adenosequences 3534 to 35938. This clone was named pWE/AflIII-rITRsp.

#### Generation of pBr/Ad.Bam-rITRspΔXba and pWE/Ad.AflIII-rITRspΔXba

Plasmid pBr/Ad.Bam-rITRsp was propagated in *E. coli* strain DM1 (*dam*<sup>-</sup>, *dcm*<sup>-</sup>) (Life Technologies). The plasmid was  
5 digested with *Xba*I, removing the 1.88-kb *Xba*I-*Xba*I fragment, and religated. The resulting clone pBr/Ad.Bam-rITRspΔXba was used to construct helper cosmid pWE/Ad.AflIII-rITRspΔXba as described above. Briefly, the following fragments were  
10 isolated by extraction from agarose gel (QIAGEN): pWE.pac digested with *Pac*I, pBr/AflIII-Bam digested with *Pac*I and *Bam*HI, and pBr/Ad.Bam-rITRΔXba digested with *Bam*HI and *Pac*I. These fragments were ligated together and packaged using  
15 lambda phage packaging extracts according to the manufacturer's instruction (Stratagene). After infection of host bacteria assembled phage, the resulting colonies were analyzed for the presence of the intact insert. pWE/Ad.AflIII-rITRspΔXba contains sequences identical to that of  
20 pWE/Ad.AflIII-rITRsp but with deletion of the *Xba*I-*Xba*I fragment.

#### Generation of pBr/Ad.Bam-rITRspΔE2AΔXba and pWE/Ad.AflIII-rITRspΔE2AΔXba

Plasmid pBr/Ad.Bam-rITRspΔE2AΔXba was constructed for the  
25 generation of E1-deleted recombinant adenoviruses with dual deletion of E2A and E3. A *Spe*I-*Bam*HI fragment containing E2A deletion was isolated from plasmid pBr/Ad.Sal-rITRΔE2A and inserted into *Spe*I-*Bam*HI-digested pBr/Ad.Bam-rITRspΔXba, yielding plasmid pBr/Ad.Bam-rITRspΔE2AΔXba. This plasmid was  
30 used to construct helper pWE/Ad.AflIII-rITRspΔE2AΔXba, using three fragment ligation as described above. pWE/Ad.AflIII-rITRspΔE2AΔXba contains sequences identical to that of pWE/Ad.AflIII-rITRsp but with dual deletions of the E2A region and the *Xba*I-*Xba*I fragment.

Generation of pBr/Ad.Bam-rITRsp $\Delta$ E3 and pWE/Ad.AflIII-rITRsp $\Delta$ E3 and of pBr/Ad.Bam-rITRsp $\Delta$ E2A $\Delta$ E3 and pWE/Ad.AflIII-rITRsp $\Delta$ E2A $\Delta$ E3

5

To allow insertion of even larger DNA fragments, an expanded deletion of the E3 region was constructed in which the complete E3 coding region was removed. Primers 1 (5'-AAA CCG AAT TCT CTT GGA ACA GGC GGC-3') (SEQ ID NO:1) and 2 (5'-GCT CTA GAC TTA ACT ATC AGT CGT AGC CGT CCG CCG-3') (SEQ ID NO:2) were used to amplify sequence from pBr/Ad.Bam-rITRsp, corresponding to sequences 27326 to 27857 in wt Ad5 genome. Primers 3 (5'-GCT CTA GAC CTC CTG TTC CTG TCC ATC CGC-3') (SEQ ID NO:3) and 4 (5'-GTA TGT TGT TCT GGA GCG GGA GGG TGC-3') (SEQ ID NO:4) were used to amplify sequence from the same DNA template, corresponding to sequences 30994 to 35502 in wt Ad5 genome. The amplification products were digested with *EcoRI/XbaI* and *XbaI/AvrII* respectively and ligated together. The resulting *EcoRI-AvrII* fragment was cloned into vectors of pBr/Ad.Bam-rITRsp and pBr/Ad.Bam-rITRsp $\Delta$ E2A that have been digested with *EcoRI* and *AvrII*, yielding pBr/Ad.Bam-rITRsp $\Delta$ E3 and pBr/Ad.Bam-rITRsp $\Delta$ E2A  $\Delta$ E3, respectively. These two plasmids were used to construct cosmid helper molecules as described above. pWE/Ad.AflIII-rITRsp $\Delta$ E3 contains sequences identical to that of pWE/Ad.AflIII-rITRsp but with a deletion of the E3 region corresponding to sequences 27857-30994 in wt Ad5 genome, while pWE/Ad.AflIII-rITRsp $\Delta$ E2A $\Delta$ E3 is identical to pWE/Ad.AflIII-rITRsp $\Delta$ E3 but with an additional deletion of the E2A region.

30

The above described cosmids are particularly useful for the production of adenoviral expression libraries in particular libraries carrying collections of large inserts. See also example 19 and 20.

## Example 19

Miniaturized, multiwell production of E1, E2A and E3 deleted  
recombinant adenoviral vectors carrying therapeutic and  
5 marker transgenes.

As mentioned in Example 10, a combined deletion of E1, E2A  
and E3 will allow cloning of foreign DNA sequence up to  
10 approximate 10.5 kb in size. Here, we show the production of  
E1, E2A and E3 deleted vectors carrying human cDNAs as well  
as marker genes in PER.C6/E2A cells.

Cell culture conditions were described in Example 10. For DNA  
15 transfection, adapter and helper molecules were prepared  
according to Example 14. Linearized adapter plasmids  
pAD/CLIP-ceNOS and pAD/CLIP-lacZ were used for transfection  
in combination with four different *PacI*-linearized helper  
cosmids, namely, pWE/Ad.AflIII-rITRsp, pWE/Ad.AflIII-  
20 rITRsp.dE2A, pWE/Ad.AflIII-rITRsp.dXba, and pWE/Ad.AflIII-rITR.  
The DNA transfection procedure was identical to that  
described in Example 10. An aliquot of 100 µl of the freeze-  
thawed lysates were used to infect a second 96-well plate  
with PER.C6/E2A cells and CPE formation was monitored. Figure  
25 35 shows the percentage of virus producing wells (CPE  
positive) in a 96-well plate of PER.C6/E2A cell after  
propagation of the freeze/thawed transfected cells. Clearly,  
it is possible to produce E1 and E3 deleted recombinant  
adenoviral vectors carrying therapeutic and marker transgenes  
30 in PER.C6/E2A cells.

## Example 20

Construction of a sense or antisense, arrayed adenoviral  
expression library for selection of phenotypes

5

The miniaturization of adenoviral vector production allows the large scale, high throughput construction, screening of cloned or pooled gene expression libraries.

10

To construct a cloned and arrayed cDNA expression library in an adenoviral vector format based on the PER.C6 (and derivatives) production system, poly(A+) mRNA of human placenta is isolated using oligo(dT) cellulose, and converted into cDNA using materials and reagents supplied by vendors such as Life Technologies Inc. (LTI; Breda, The Netherlands).

15

The resulting double stranded cDNA molecules contain a SalI-compatible overhang at the 5' end and a NotI-compatible overhang at the 3' end. The total cDNA was ligated into pIPspAdapt6 (for sense orientation of cDNA inserts) or pIPspAdapt7 (for antisense orientation of cDNA inserts) (see

20

example 17 for adapter configurations). For this, pIPspAdapt6 and pIPspAdapt7 were digested with the restriction endonuclease MluI, followed by dephosphorylation of the 5' overhangs using thermosensitive alkaline phosphatase (LTI). After digestion with SalI and NotI, the linearized plasmid

25

was isolated on a 0.8% agarose gel and purified by anion exchange chromatography. Following ligation of the cDNA molecules into the plasmids, the resulting library was introduced into *E. coli* DH5a electrocompetent bacteria by electroporation on a BTX 600 electrocell manipulator or equivalent. The unamplified library was aliquoted and frozen as glycerol stock.

30

On the day of plating, vials were thawed and plated on large petridishes containing LB medium with 1.5% agar and ampicillin at 50 micrograms per ml. To obtain even distribution of the plated colonies, glass beads were used

35



while plating. After over night growth at 37 °C, the agar-plates were transferred to an automated colony picking robot (Flexys; Genome solutions. Individual colonies were picked and transferred by the robot to microtiter plates with 300 µl of Terrific Broth medium and ampicillin at 50 micrograms per ml. Plates inoculated in this way are then transferred to HiGro incubators (Genemachines) aerated with oxygen and grown according to the manufacturers manual for 12-16 hours.

Thereafter, the individual plasmids were isolated by the conventional alkaline lysis plasmid DNA isolation method as described in Sambrook et al. (Sambrook, J. et al, eds. (1989) Molecular cloning: a laboratory manual, 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press). For this, the plates are first transferred to centrifuges (e.g. Eppendorf 5810R or Heraeus Megafuge 2.0) and bacteria are pelleted for 20 minutes at 1500xg. Using liquid robotic handlers the supernatant in the individual wells of the individual plates is removed and discarded. Bacterial pellets are resuspended in 100 µl 25 mM Tris, pH 8.0, containing 50 mM glucose and 10 mM EDTA, and bacteria are lysed by adding 100 µl of 0.2N NaOH/1% SDS. Following neutralization by adding 100 µl of 5M potassium acetate, a cleared lysate is obtained by filtration over a MultiScreen-NA lysate clearing plate (Millipore B.V., Etten-Leur) or equivalent thereof, using a vacuum manifold.

The plasmid DNA in the cleared lysate is subsequently precipitated by adding 200 µl of 2-isopropanol and centrifugation at 1500xg for 30 minutes at 4°C. The precipitate is washed once with 70% ethanol and, after air drying, taken up in 20 µl of TE.

The isolated plasmid DNA in each individual well is quantified using the Picogreen DNA quantification kit as described by Molecular Probes (Eugene, Oregon, USA) by transferring an aliquot of the plasmid DNA from each well to fresh plates with the appropriate dilution. In the mean time PER.C6 cells, or derivatives such as PER.C6/E2A, are seeded

as described under the other examples for miniaturized adenovirus generation. For each well, 55 nanogram of purified plasmid DNA was transferred into a new plate and linearized with PiPspI for 60 minutes at 65°C. This plasmid is then  
5 cotransfected with an appropriate helper DNA molecule (e.g. E2A deleted (such as pWE/Ad.AflIII-rITR.deltaE2A) or E2A/E3 deleted or E2A/E3/E4 deleted, see example 18) into PER.C6 or PER.C6/E2A packaging cells. Transfection is similar to the experiments and methods described in examples 9,10 or 25 for  
10 adenovirus generation in mikrotiter plates. Virus formation in individual wells is quantified using CPE formation, blot based virus assays or reporter systems. The arrayed adenovirus library is then ready to be used in cell based screens where one can select for a particular  
15 phenotype.

In figure 36 an overview is given of the scheme of an adenoviral cDNA expression library constructed and arrayed as described above. This scheme describes the construction of  
20 libraries of individually cloned adenoviral vector libraries in a high throughput fashion. The improvement of this strategy over pooled libraries is that no bias for viruses with a growth advantage can occur. This is because individual members of the library are in the format of individual  
25 colonies straight after the plating of the library, and are kept individually during all further procedures.

The adenoviral expression library can be used for infection of different cells appropriate for selection of a particular  
30 phenotype such as capillary formation, cell proliferation, cell migration or marker gene expression either in an appropriate unmodified cell type or a reporter cell line designed for this purpose. Detection of these phenotypes can be done for example using automated image analysis of  
35 morphology changes or changes in intracellular localization of a reporter protein. Once hits have been selected, the

cloned bacterial DNA version is available immediately for sequence analysis in the form of the pIPspAdapt6 or pIPspAdapt7 adapter plasmid as produced in *E. coli*. This means that no rescue is necessary as is the case with pooled retroviral or plasmid-based expression libraries.

If desired, (for example using liquid handler robots or manually), individual wells containing individual adenoviral vectors in one row or one column or one plate or multiple plates can be pooled before doing assays. This is the case if a desired assay is not amenable to high throughput analyses and the total number of wells needs to be decreased for a primary screen. An additional improvement or advantage of pooled but originally cloned adenoviral vectors is that multi-gene dependant phenotypes are selectable.

#### Example 21

Virus production in wells of a 384 well tissue-culture plate

Essentially this experiment has been performed as described in example 10, except for the following minor changes. The day before transfection, PER.C6/E2A cells were diluted with culture medium (DMEM with 10% fetal bovine serum and 10 mM MgCl<sub>2</sub>) to a suspension of 11.250 cells per 25 µl followed by seeding 25 µl per well of the 384-well-tissue-culture plate using a 16 channel multichannel pipette (Finn). After adding 1.3 ml serum free DMEM to the DNA/lipofectamine mixture, 15 µl per well of this mixture was then added to PER.C6/E2A seeded wells that were washed with 25 µl DMEM prior to transfection. After 3 hours in a humidified CO<sub>2</sub> incubator (39°C, 10% CO<sub>2</sub>) 50 µl culture medium was added to each well and the plates were returned to the humidified CO<sub>2</sub> incubator (39°C, 10% CO<sub>2</sub>). The next day the medium of each

well was replaced with 50  $\mu$ l culture medium. The plates were then returned to a humidified CO<sub>2</sub> incubator (32°C, 10% CO<sub>2</sub>) for an additional 4 days after which the wells were subjected to freezing at -20°C overnight followed by thawing and  
5 resuspension by repeated pipetting. An aliquot of 25  $\mu$ l of the freeze/thawed transfected cells was transferred to each well of a plate with fresh PER.C6/E2A cells seeded as described above on 384-well-tissue culture plates (plate 2). The second 384-well plate with PER.C6/E2A cells incubated and  
10 thus infected with freeze/thawed cell lysate of the first transfected plate was checked for CPE formation and stored at -20°C. The experiment mentioned above was performed twice. In **figure 37** the percentage CPE positive wells scored after propagation of the freeze/thawed transfected cells to new  
15 PER.C6/E2A cells, is depicted.

#### Example 22

20 The Effect of omitting propagation or refreshment of culture medium instead of propagation, on the speed and production efficiency of virus formation

Making the process of miniaturized adenoviral vector  
25 production more amenable to automation calls for a simplification of the whole procedure. One laborious and time consuming step is the propagation of cell lysates from transfected PER.C6/E2A cells on fresh cells and therefore omitting of this step is desirable. The following experiments  
30 to reach this goal. In order to determine the effect of changing the medium of transfected cells instead of using the freeze/thawed, transfected PER.C6/E2A cells (see example 10 and others) to infect new PER.C6/E2A cells (propagation) or omit propagation all together, the following experiment was  
35 performed. The day before transfection, PER.C6/E2A cells were

harvested using trypsin-EDTA and counted. The cells were then diluted with culture medium (DMEM with 10% fetal bovine serum and 10 mM MgCl<sub>2</sub>) to a suspension of 22.500 cells per 100 µl, followed by seeding 100 µl per well of the 96-well-

5 tissueculture plates. The next day 2.6 µg of the linearized adapter molecules and 2.6 µg of the PacI linearized pWE-Ad.AflIII-rITRdE2A plasmid DNA, in a volume of 100 µl serum free Dulbecco's Modified Eagles Medium (DMEM, were mixed with 26.5 µl lipofectamine diluted in 74.4 µl serum free DMEM by

10 adding the lipofectamine mix to the DNA mix. The DNA/lipofectamine mixture was left at room temperature for 30 minutes, after which 1.3 ml serum free DMEM was added. The latter mixture was then added (30 µl per well) to PER.C6/E2A seeded wells that were washed with 200 µl DMEM prior to

15 transfection. All of the transfections were performed in duplicate. After three hours in a humidified CO<sub>2</sub> incubator (39°C, 10% CO<sub>2</sub>) 200 µl culture medium was added to each well and the plates were returned to the humidified CO<sub>2</sub> incubator (39°C, 10% CO<sub>2</sub>). The next day the medium of each well was

20 replaced with 200 µl culture medium. The plates were then returned to a humidified CO<sub>2</sub> incubator (32°C, 10% CO<sub>2</sub>). After seven days, the medium of one of the two transfected plates was replaced with 200 µl culture medium and returned to a humidified CO<sub>2</sub> incubator (32°C, 10% CO<sub>2</sub>), after which the

25 forming of CPE was followed. In figure 38A the percentage of virus producing cells (CPE positive wells), scored after changing the medium of the transfected cells instead of propagation and amplification fresh PER.C6/E2A cells, is depicted.

30 The wells of the second plate were subjected to freezing at -20°C overnight, followed by thawing and resuspension by repeated pipetting. An aliquot of 100 µl of the freeze/thawed transfected cells was transferred to each well of a plate with new PER.C6/E2A cells (2.25x10<sup>4</sup> cells per well in 100 µ

1), seeded in 96-well-tissue-culture-plates one day prior to infections. This was incubated in the humidified CO<sub>2</sub> incubator (32°, 10% CO<sub>2</sub>) until the presence of full CPE was observed. In figure 38B, the percentage of virus-producing cells (CPE positive wells) scored after propagation on fresh PER.C6/E2A cells, is depicted. In all experiments untransfected wells were included for control of cross contamination. All these wells remained negative for CPE formation. In figure 38C the results of a parallel normal procedure as described under example 10 are given. These results show that replacement of medium or complete omitting of any handling after transfection can replace reinfection of fresh PER.C6/E2A cells with lysate from the primary transfectant plates.

15

#### Example 23

Determination of the influence of the cell growth of PER.C6/E2A cells on the speed and production efficiency of virus formation

20

For construction of adenoviral gene expression libraries the conditions for miniaturized production of adenoviral vector need to be optimal. For this purpose a number of parameters that may influence virus generation were varied and their effect in adenoviral vector production measured.

25

In order to determine how the cell confluency of the complementing cell line PER.C6/E2A prior to seeding in mikrotiter plates influences the speed and efficiency of virus production, the following experiment was performed. On day one PER.C6/E2A cells were harvested using trypsin-EDTA and counted, followed by seeding 1/10, 1/5 and 1/2.5 of the harvested cells in three different 175 cm<sup>2</sup>-tissue-culture flasks. In table 9 the number of cells, that were seeded in each 175-tissue-culture flask in three different experiments, are shown. Four days later the PER.C6/E2A cells from each

30

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flask were harvested, counted and then diluted with culture medium (DMEM with 10% fetal bovine serum and 10 mM  $MgCl_2$ ) to a suspension of 22.500 cells per 100  $\mu l$ . From each cell suspension two 96-well-tissue culture plates were seeded with 100  $\mu l$  cell suspension per well. The next day 10.6  $\mu g$  of SalI linearized pAd/Clip-lacZ and 10.6  $\mu g$  of the PacI linearized pWE-Ad.AflII-rITRdE2A plasmid DNA, in a volume of 600  $\mu l$  serum free Dulbecco's Modified Eagles Medium (DMEM) were mixed with 153.6  $\mu l$  lipofectamine diluted in 446.4  $\mu l$  serum free DMEM by adding the lipofectamine mix to the DNA mix. The DNA/lipofectamine mixture was left at room temperature for 30 minutes, after which 7.8 ml serum free DMEM was added. The latter mixture was then added (30  $\mu l$  per well) to PER.C6/E2A seeded wells that were washed with 200  $\mu l$  DMEM prior to transfection. After three hours in a humidified  $CO_2$  incubator (39°C, 10%  $CO_2$ ) 200  $\mu l$  DMEM with 10% fetal bovine serum and 10 mM  $MgCl_2$  was added to each well and the plates were returned to the humidified  $CO_2$  incubator (39°C, 10%  $CO_2$ ). The next day the medium of each well was replaced with 200  $\mu l$  DMEM with 10% fetal bovine serum and 10 mM  $MgCl_2$ . The plates were then returned to a humidified  $CO_2$  incubator (32°C, 10%  $CO_2$ ). After two days, one of the two transfected plates was used to determine the transfection efficiency using lacZ staining. In table 9, the transfection efficiency of each 96-well-tissue-culture plate scored after lacZ staining in three different experiments, is shown. The second plate of the two transfected plates was used for virus production. Seven days after transfection the wells of the second plate were subjected to freezing at -20°C overnight followed by thawing and resuspension by repeated pipetting. An aliquot of 100  $\mu l$  of the freeze/thawed transfected cells was transferred to each well of a plate with new PER.C6/E2A cells ( $2.25 \times 10^4$  cells per well in 100  $\mu l$ ), seeded in 96-well-tissue-culture-plates one day prior to infections. This was incubated in the humidified  $CO_2$  incubator (32°, 10%  $CO_2$ ) until the presence of full CPE was observed. In figure 39, the percentage of virus

producing cells (CPE positive) wells scored after propagation of the freeze/thawed transfected cells to new PER.C6/E2A cells, is depicted. The data indicate that the level of confluency of the PER.C6/E2A cells prior to transfection with the adenoviral adapter and helper DNA molecules influences the final percentage of virus producing wells the higher confluency being the most optimal for absolute final number of wells producing virus and also the speed at which the virus generation occurs.

#### Example 24

Long-term incubation with adenoviral supernatant allows detection of slow phenotypes

The use of adenoviral vector libraries in functional genomics calls for the use of appropriate cell based assays which are amenable to HTS and miniaturization in addition to a phenotype that is detectable and relevant for the genes one is looking for such as the ones used in example 12. The time of assaying after infection with an adenoviral expression library, for example in a setup such as described in example 20, is variable and depending on the parameters determining the phenotype being assayed for. For example using automated image analysis, the formation of blood capillaries in each well can be assayed simply by detecting the formation of capillaries. Formation of these structures which are indicative for angiogenesis or blood vessel formation can be induced by infection of relevant precursor cells. Such cells can be endothelial cells from heart or tumor origin, with an adenoviral vector carrying a relevant transgene for example a vascular endothelial like growth vector (VEGF). However a complex phenotype such as capillary formation only appears after several days to weeks. Therefore expression of the library of genes as mediated by the adenoviral expression



cassette in some cases needs to be long enough for allowing the phenotype to develop. In figure 40 the results are shown of an experiment with an EGFP-adenoviral vector which was used to infect A549 cells in 96 well plates. Based on the stability features described in example 15 the adenoviral dilution (in DMEM) was not removed but left for a up to 2 weeks and EGFP expression measured at regular intervals. Clearly these experiments show that adenoviral transduction can be regarded as semi-stable and even increases over time suggesting that reinfection occurs and/or infection of newly divided cells. This implies that the transient adenoviral vector system can be used to screen for phenotypes that take 2 weeks or more to develop by leaving the adenoviral supernatant on the cells in the multiwell plates (96,384 well or smaller).

#### Example 25

Miniaturized, multiwell production of recombinant adenoviral vectors using cost effective polyethylenimine (PEI) as DNA transfection agent

For the purpose of cost reduction and variable toxicity reduction it is desirable to replace the liposomal transfection reagent lipofectamine. The cationic polymer polyethylenimine (PEI) has been tested for this purpose. in the miniaturized, multiwell (96-well) adenoviral vector production system. See also example 9 and 10. PEI has been tested for transfection of PER.C6 as well as PER.C6/E2A, with different transgene inserts in the adenoviral helper plasmid: *LacZ* and *EGFP*. Different parameters were tested: PEI/DNA ratios, incubation times amounts of PEI/DNA complex per single well.

#### Testing of PEI with different PEI/DNA ratios

The 96 well microtiter plates were seeded the day before transfection with PER.C6 or PER.C6/E2A cells as described in

example 10. The next day, for 16 wells (8 wells in duplicate on different plates), 3 micrograms of *SalI* linearized pCLIP-LacZ and 3 micrograms *PacI* linearized pWEAflIIrITR for PER.C6 and pWEAflIIrITRdE2A for PER.C6/E2A were diluted in 150  $\mu$ l 150 mM NaCl and incubated at RT for 10 min. Also a 20 mM 25kDa PEI solution was diluted in 150  $\mu$ l 150 mM NaCl at different amounts to obtain different PEI/DNA ratio's and incubated at RT for 10 min. See table 4.

10 DNA and PEI solution were mixed by adding PEI by drops to DNA and incubated for 10 min at RT. Cells were washed with 100  $\mu$ l of serum free Dulbecco's Modified Eagles Medium (DMEM)/ well. Then 1.3 ml of DMEM was added to the mix and 80  $\mu$ l per well was applied to the cells in each well. As a positive control  
15 DNA/lipofectamine complexes were transfected (prepared according to example 9). Additional control incubations were only DMEM, only PEI without DNA (ratio 13) and twice the amount of PEI/DNA ratio 13 were included. After 4 hrs of incubation at 37°C for PER.C6 and at 39°C for PER.C6/E2A in a  
20 humidified CO<sub>2</sub> incubator, for the PEI transfections 80  $\mu$ l of PEI/PER.C6 medium (DMEM with 20 % v/v fetal calf serum (FCS) 10 mM MgCl<sub>2</sub>)/well was added to the cells. For the lipofectamine transfections 180  $\mu$ l of DMEM 10 % v/v FCS 10 mM MgCl<sub>2</sub>/well was added to the cells and the plates were  
25 returned to the humidified CO<sub>2</sub> incubator. The next day the medium of each well was replaced with 200  $\mu$ l DMEM 10 % v/v FCS 10 mM MgCl<sub>2</sub>. The plates were then left at 37°C for PER.C6 plates and at 32°C for PER.C6/E2A plates in a humidified CO<sub>2</sub> incubator. After 3 days one of the duplicate plates was  
30 stained with X-gal to determine the transfection efficiency. The transfection efficiency results are depicted in table 5. After four days post-transfection, The plates were subjected to freezing at 20°C for 4 hrs followed by thawing and resuspension by repeated pipetting. An aliquot of 100  
35 microliters was transferred to a new plate of PER.C6 or PER.C6/E2A cells seeded as described above a day before. The

plates were then placed back into the CO<sub>2</sub> incubators. After 14 days post-propagation, CPE as an indicator for virus formation was scored and the plates were subjected to freezing at 20°C for 4 hrs followed by thawing and resuspension by repeated pipetting. An aliquot of 20 µl was transferred to wells of plates seeded with 1.10<sup>4</sup> A549 cells per well of a 96 well plate in a volume of 100 µl. Two days after infection the wells were stained with X-Gal for LacZ activity as described under example 9. The results are summarized in table 6 .

Testing of PEI with different PEI/DNA ratio's in combination with different amounts of complex per well

In order to test the optimum, absolute amount of PEI/DNA complex at two ratios which can be applied to the cells, without being toxic, the PEI/DNA ratio's 5 and 11.7 were tested. This was tested on PER.C6/E2A. The standard concentration (1X) is the concentration as described above in the previous transfection experiment (see table 4).

To make PEI solutions with amounts of PEI between 0.9 and 42 µl, various amounts of a 150 mM NaCl solution were added to the 20 mM 25 kDa PEI (Fluka cat.nr.03880) solution to a final volume of 300 µl. From this solution 150 microliters was added to the DNA mix (see table 7).

DNA (50% pCLIP-LacZ and 50% pWEAflIIrITRdE2A) to 150 microliters with 150 mM NaCl.

Transfections were performed as described above.

Lipofectamine was used as a positive control as well as DNA or PEI or DMEM without any additives. After three days a duplicate plate was stained for lacZ expression and the results are given in table 8. First, the cells were checked for toxicity. A difference could be seen between the two ratios. At ratio 11.7 the double concentration (2 X) is more toxic, but the transfection efficiency is higher as X. At concentration 0.1 X for both ratios no blue cells were seen

after staining, indicating that the cells were not transfected.

Processing, CPE monitoring, A549 transduction and lacZ staining was done as described.

To test toxicity quantitatively the latter transfections were repeated and two days after medium replacement a cell proliferation assay (Promega) was used to determine the numbers of living cells or of toxicity of PEI/DNA complexes.

All actions were according to the manufacturers protocol.

After 4 hrs of incubation at 37°C the plates were read using the microplate manager (Bio-Rad). The results of this experiment for PEI ratio 5 and 11.7 are summarized in figure 41. Clearly toxicity is lowest and virus generation optimal at ratio 5 (1.5 times the standard amount of complex) and at ratio 11.7 at the standard conditions (between 0.5 and 1.5 times the standard amounts).

Testing of PEI as DNA carrier with different PEI/DNA ratios, with a different gene and warm vs. cold PEI.

In order to test if the temperature of PEI influences the complex formation, the above described protocol was tested with PEI at 4 C and at RT. In addition, another transgene insert was tested; EGFP. Also the best concentrations of the two ratios were used in this transfection experiment (450 ng DNA/well PEI/DNA ratio 5 and 300 ng DNA/well PEI/DNA ratio 11.7). Processing, CPE monitoring, A549 transduction and lacZ staining was done as described and as can be seen in figure 42 there's no significant difference observed between warm and cold PEI. Virus formation with EGFP and PEI worked very well (PEI ratio 5 warm and the positive control lipofectamine both 100 % CPE).

Testing of PEI as DNA carrier with different PEI/DNA complex volumes per well.

5 In order to test if the volume of DNA/PEI complex influenced the efficiency of virus generation in the above described protocol, different volumes of the PEI/DNA complex (ratio 5 450 ng DNA per well, PEI 20 mM 25kDa Fluka) were added to the cells. In this case 30, 80 and 120 microliters per well was  
10 added. Processing, CPE monitoring, A549 transduction and lacZ staining was done as described above. As can be seen in figure 43, there's a significant difference in transfection efficiency between applying 30  $\mu$ l, 80  $\mu$ l, and 120  $\mu$ l. Using 30  $\mu$ l only 1% of the cells within a well was stained blue,  
15 whereas for 80  $\mu$ l 60% of the cells stained blue. Increasing the amount of complex to 120  $\mu$ l resulted in the same results as applying 80  $\mu$ l. For virus formation the same trend was observed; no CPE was found for 30  $\mu$ l, whereas 80 and 120  $\mu$ l gave similar percentages (not shown). In conclusion PEI can  
20 be used to used to produce adenoviral vectors in a miniaturized setup.

Example 26

Miniaturized, multiwell production of recombinant adenoviral  
25 vectors without a cell washing step prior to transfection.

For the purpose of reducing steps in automation of the miniaturized, multiwell production of recombinant adenoviral vectors and cost reduction, the serum free medium (SFM)  
30 washing step of the PER.C6 or PER.C6/E2A cells or derivatives prior to transfection was removed from the standard protocol. Transfections were performed as described in example 10. The transfections were performed using the human ceNOS as transgene insert and Removal of the cell washing step was  
35 tested and compared to the standard procedure with washing. Processing and CPE monitoring was done as described and as

can be seen in figure 44 there's no significant reduction observed in virus production in case the cells were not washed prior to transfection.

5 In conclusion removal of the cell washing step to remove the bulk of serum proteins as part of the standard transfection protocol is possible without effecting the CPE efficiency. The latter is very useful when reducing the complexity of the whole process desirable for when automating the miniaturized, multiwell production of recombinant adenoviral vectors.

10

## EXAMPLE 27

THE USE OF ADENOVIRAL CONSTRUCTS TO MODULATE GENE EXPRESSION IN ZEBRAFISH.

15

Modulation of gene expression by adenoviral constructs in whole animals can give important information about the function of genes. For instance, adenoviral constructs that express a sense cDNA construct encoding a full length protein can be used for the over-expression of that protein in animal model systems, while adenoviral constructs that express the antisense cDNA can be used to reduce the expression levels of the endogenous protein. In addition, over-expression of an adenoviral-encoded protein might rescue a mutant phenotype.

25 Adenoviral-mediated modulation of gene expression in animal models can give important information about the function of a gene.

In this example, zebrafish, *Danio rerio*, will be discussed as an animal model system to show the feasibility of the approach. To this end, zebrafish cDNA libraries will be screened with cDNA's that are identified and isolated by methods described in this application. The thus obtained homologous zebrafish cDNA's, encoding full length proteins, will be isolated and cloned in both orientations, sense and

30

antisense, in adapter plasmids of the pIPSPAdapt series (see example 17). These will subsequently be used to generate recombinant adenovirus, which will be used to infect either wildtype or mutant (see for instance *Development* 1996 Volume 123, December) zebrafish embryos. Methods for breeding zebrafish are well known to those skilled in the art.

The effect of up- or down-modulation of gene expression can be studied in wildtype or mutant embryos or adult fish.

10 Embryos will be collected as follows: Zebrafish are photo-periodic in their breeding and produce embryos every morning shortly after sunrise. For continuous production of a relatively small number of embryos (30-50 per tank per day) an equal number of males and females are used. The day-night

15 cycle is controlled with an automatic timer (14 hr light/10 hr dark). The bottom of the tank is covered with a single layer of marbles to keep the fish from eating the newly spawned eggs. Freshly produced embryos are collected each morning by siphoning the bottom of the tank and infected with

20 recombinant adenovirus. This can be achieved in several ways, as described below. The method of choice depends on the expression pattern of the gene.

Recombinant adenovirus can be injected directly into the

25 chorion fluid, after which the embryos are washed and cultivated further in system water.

Similarly, recombinant adenovirus can be deposited at specific sites in embryos or adult fish, for instance by injection into the blood stream, or by oral or rectal

30 administration. Injection can be performed by holding the embryos in wedged-shaped troughs made with a plastic mold in 1.5% agarose, in which case there is no need to remove their chorions. Each trough can hold approximately 35 embryos (with chorions). Embryos can be aligned by gently tapping them down

with forceps. Agarose is useful because pipette tips generally will not break if they accidentally touch the surface. As the pipette penetrates the chorion, the embryo is forced against the rear vertical wall of the trough. The exact positioning of the pipette tip within the embryo is achieved by slight movement of the pipette with a micro-manipulator or by movement of the stage. Alternatively, embryos can be dechorionated (see below) and incubated in medium containing recombinant adenovirus.

10

After injection, 25-30 eggs will be deposited into 250 ml beakers. After hatching, larvae will be transferred into a new beaker and completely separated from their chorions. Larvae are raised under standard conditions well known to those skilled in the art.

15

Monitoring changes after adenoviral infection of zebrafish can be done as early as the embryonic stage. Some observations of zebrafish development can be made directly through the chorion. However, for most procedures it is better to remove the chorion. Chorions can be removed easily with sharp forceps. When raised at 28.5°C, zebrafish develop normally outside their chorions. Embryos removed from their chorions can be transferred from one container to another by gently pipetting them up with a fire-polished Pasteur pipette or by gentle pouring. Small petri-dishes (35 mm diameter) are adequate for holding up to 25 embryos during the first few days of development. The embryos can be brought to the center of the dish for viewing by gently swirling the medium in a circular motion.

20  
25  
30

Larvae and adult fish can be monitored without further treatment.

More elaborate analysis methods include the staining of sections by classical histological methods, or by using



specific methods such as anti-sense hybridization or incubation with antibodies to look at differences at the molecular level.

- 5 The phenotypic changes that are observed after infection of zebrafish with recombinant adenovirus can give important information about the function of the encoded genes *in vivo*. The method described above can also be applied to other animal models.

Table 4

Ratio	# 20 mM PEI ( $\mu$ l)	# 150 mM NaCl ( $\mu$ l)
8.3	7.5	142.5
10	8	142
11.7	10.5	139.5
13	12	138
15	13.5	136.5

5 Table 5 Transfection efficiency control. X-gal staining.

Ratio	% blue cells PER.C6	% blue cells PER.C6E2A
8.3	45	60
10	45	60
11.7	55	65
13	55	65
15	50	40
2*13	10	10
Only PEI 13	0	0
Only DMEM	0	0
LIPO	65	80

141

Table 6

PER.C6 ratio	#CPE	%CPE	# blue wells after infection A549	% blue wells after infection A549
8.3	6/8	75	7/8	87.5
10	3/8	37.5	5/8	62.5
11.7	4/7	57	4/7	57
13	4/8	50	5/8	62.5
15	3/7	37.5	3/7	43
2*13	0/8	0	0/8	0
Only PEI 13	0/8	0	0/8	0
Only DMEM	0/8	0	0/8	0
Lipofectamine	1/16	6	1/8	12.5

PER.C6/E2A ratio	#CPE	%CPE	# blue wells A549 cells	% blue A549 cells
8.3	0/8	0	0/8	0
10	1/8	12.5	3/8	37.5
11.7	3/8	37.5	6/8	75
13	1/8	12.5	2/8	25
15	1/8	12.5	2/8	25
2*13	0/8	0	0/8	0
Only PEI 13	0/8	0	0/8	0
Only DMEM	0/8	0	0/8	0
Lipofectamine	11/16	69	13/16	81

Table 7

Concentration of PEI/DNA complex	Amount of DNA/well (ng/ $\mu$ l)	DNA ( $\mu$ g)	PEI ratio 11.7 ( $\mu$ l)	PEI ratio 5 ( $\mu$ l)
2 X	600	12	42	18
1.5 X	450	9	31.5	13.5
Standard 1 X	300	6	21	9
0.5 X	150	3	10.5	4.5
0.1 X	30	0.6	2.1	0.9

5 Table 8

Complex	Amount of DNA (ng) per well	% blue cells PEI ratio 5	% blue cells PEI ratio 11.7
PEI 2 X	600	30	45
PEI 1.5 X	450	40	55
PEI standard X	300	25	Not determined
PEI 0.5 X	150	5	40
PEI 0.1 X	30	0	0
Lipofectamine	100	65	65
-/-	0	0	0

Table 9

Confluencies of cells harvested for transfection

T <sub>175</sub> flask	Cell# 1 <sup>st</sup> exp.	Cell# 2 <sup>nd</sup> exp.	Cell# 3 <sup>rd</sup> exp.
1/10	2.3x10 <sup>6</sup>	1.3x10 <sup>6</sup>	3.3x10 <sup>6</sup>
1/5	4.7x10 <sup>6</sup>	2.6x10 <sup>6</sup>	6.7x10 <sup>6</sup>
1/2.5	-	5.2x10 <sup>6</sup>	13.3x10 <sup>6</sup>

Transfection efficiencies

96-well-plate	Efficiency 1 <sup>st</sup> exp.	Efficiency 2 <sup>nd</sup> exp.	Efficiency 3 <sup>rd</sup> exp.
1/10	30-40%	50-60%	50-60%
1/5	70-80%	50-60%	50-60%
1/2.5	-	50-60%	50-60%

5

10 All publications and patent applications mentioned in  
 this specification are indicative of the level of skill of  
 those skilled in the art to which this invention pertains.  
 All publications and patent applications are herein  
 incorporated by reference to the same extent as if each  
 15 individual publication or patent application was specifically  
 and individually indicated to be incorporated by reference.

The invention now having been fully described, it will  
 be apparent to one of ordinary skill in the art that many  
 20 changes and modifications can be made thereto without  
 departing from the spirit or scope of the appended claims.

25

144

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

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(ii) TITLE OF INVENTION: HIGH THROUGHPUT SCREENING OF GENE  
FUNCTION USING ADENOVIRAL LIBRARIES FOR FUNCTIONAL  
GENOMICS APPLICATIONS

15

(iii) NUMBER OF SEQUENCES: 47

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US NOT ASSIGNED  
(B) FILING DATE: 12-JUN-1998  
(C) CLASSIFICATION:

35

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45

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: other nucleic acid

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTGTCTTA ATTAACCGCT TAA

23

145

## (2) INFORMATION FOR SEQ ID NO:2:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATTGTCTTA ATTAACGC

19

(2) INFORMATION FOR SEQ ID NO:3:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAITGCGGTT AATTAGAC

19

35

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 47 base pairs  
40 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

50

CTGTACGTAC CAGTGCACTG GCCTAGGCAT GGAAAAATAC ATAAC TG

47

(2) INFORMATION FOR SEQ ID NO:5:

- 55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 64 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

146

(ii) MOLECULE TYPE: other nucleic acid

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

10 GGGGATCCCTT CGAACCATGG TAAGCTTGGT ACCGCTAGCG TTAACCGGGC GACTCAGTCA 60  
ATCG 64

(2) INFORMATION FOR SEQ ID NO:6:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGCCACCAT GGGCAGAGCG ATGGTGGC 28

30 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
35 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

45 GTTAGATCTA AGCTTGTCGA CATCGATCTA CTAACAGTAG AGATGTAGAA 50

(2) INFORMATION FOR SEQ ID NO:8:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: other nucleic acid



147

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGTATTAGG CCAAAGGCGC A

21

5 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

20 GATCCCATGG AAGCTTGGGT GGCGACCCCA GCG

33

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: other nucleic acid

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATCCCATGG GGATCCTTTA CTAAGTTACA AAGCTA

36

(2) INFORMATION FOR SEQ ID NO:11:

40

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

45 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

55 GTCGCTGTAG TTGGACTGG

19

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

148

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5       (ii) MOLECULE TYPE: other nucleic acid

10       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGATAAGCTT AATTCCTTTG TGTTT

25

(2) INFORMATION FOR SEQ ID NO:13:

15

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: other nucleic acid

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CITAGGTAAAC CCAGTAGATC CAGAGGAGTT CAT

30

33

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 63 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: other nucleic acid

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

45

AACTGCAGAT CTATCGATAC TAGTCAATTG CTCGAGTCTA GACTACGTCA CCGCCCCCGT  
TCC

60

63

50       (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: other nucleic acid

149

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGGGATCCGT CGACGCGGCC GCATCATCAA TAATATACC

39

(2) INFORMATION FOR SEQ ID NO:16:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: other nucleic acid

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGATGCATCG

10

25 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

40 GGGGTGGCCA GGGTACCTCT AGGCTTTTGC AA

32

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45

50 (ii) MOLECULE TYPE: other nucleic acid

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGGGGATCC ATAAACAAGT TCAGAATCC

29

(2) INFORMATION FOR SEQ ID NO:19:

150

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCTTGAATT CCCGGGTACC T

21

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTAGAGGTAC COGGAATTC A

21

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGGAATTCCTT AATTAGTTA ACATCATCAA TAATATACC

39

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 66 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

151

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ACGGCGCGCC TTAAGCCACG CCCACACATT TCAGTAGTGA CTAGTCTACG TCACCGGCCC 60  
CGTTCC 66

10 (2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

25 CGGAATTCAT CAGGATAGGG CGGTGG 26

(2) INFORMATION FOR SEQ ID NO:24:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: other nucleic acid

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGGGATCCTA TGATATTTA AATGTTTTAG GGCGGAGTAA CTTG 44

45 (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

152

TAAGCCACTA GTACGTACTG AAATGTGTGG GCGTGGC

37

(2) INFORMATION FOR SEQ ID NO:26:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 43 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTAAGCCACG CCCACACATT TCAGTACGTA CTAGTGGCCT AAT

43

20 (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

35 CGGTGTAGTGT ATTTATACCC G

21

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:  
40 (A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TCGTCACTGG GTGGAAAGCC A

21

(2) INFORMATION FOR SEQ ID NO:29:

- 55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

153

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

10 TACCGCCCGT CCTAAAATGG C

21

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GCCTCCATGG AGGTCAGATG T

21

(2) INFORMATION FOR SEQ ID NO:31:

30

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

45 GCTTGAGCCC GAGACATGTC

20

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

154

CCCCCTCGAGC TCAATCTGTA TCCT

24

(2) INFORMATION FOR SEQ ID NO:33:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

10

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGGGGATCCG AACTTGTTTA TTGCAGC

27

20 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

25

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGGAGATCTA GACATGATAA GATAC

25

35

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

40

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

50

GGGAGATCTG TACTGAAATG TGTGGGC

27

(2) INFORMATION FOR SEQ ID NO:36:

55

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear



155

(ii) MOLECULE TYPE: other nucleic acid

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGAGGCTGCA GTCTCCAACG GCGT

24

10

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGCGAATTCG TCGACATCAT CAATAATATA CC

32

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

30

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

40

CTGTGTACAC CGGCGCA

17

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

45

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

55

GTACACTGAC CTAGTGCCGC CCGGGCAAAG CCGGGGCGGC ACTAGGTCAG

50

(2) INFORMATION FOR SEQ ID NO:40:

156

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:
- GTACCTGACC TAGTGCCGCC CGGGCTTTCG CCGGGCGGCA CTAGGTCAGT
- (2) INFORMATION FOR SEQ ID NO:41:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 55 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:
- GTACATTGAC CTAGTGCCGC CCGGGCAAAG CCGGGCGGC ACTAGGTCAA TCGAT
- (2) INFORMATION FOR SEQ ID NO:42:
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(A) LENGTH: 55 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
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- GTACATCGAT TGACCTAGTG CCGCCCGGGC TTGCCCCGGG CGGCACTAGG TCAAT
- (2) INFORMATION FOR SEQ ID NO:43:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
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- TGGACTTGAG CTGTAAACGC
- (2) INFORMATION FOR SEQ ID NO:44:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid

157

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GGGGGATCCT CAAATCGTCA CITCCGT

27

- (2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
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  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GGGGTCTAGA CATCATCAAT AATATAC

27

- (2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GGCGAATTCG GTACCATCAT CAATAATATA CC

32

- (2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
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  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GTACACTGAC CTAGTGCGC CCGGGCAAAG CCGGGCGGC ACTAG

45

Claims

1. A library of expressible nucleic acids comprising a multiplicity of compartments, each comprising at least one vehicle comprising at least one nucleic acid of said library, whereby said vehicle is capable of very efficiently  
5 introducing said at least one nucleic acid in a cell such that it can be expressed.
2. A library according to claim 1, wherein said vehicle comprises a viral element or a functional part, derivative and/or analogue thereof.
- 10 3. A library according to claim 1 and claim 2, wherein said viral element is derived from an adenovirus.
4. A library according to anyone of claims 1-3, wherein said cell is a eukaryotic cell, preferably a mammalian cell.
5. A library according to anyone of claims 1-4, wherein  
15 at least one compartment comprises at least two of said at least one vehicle.
6. A library according to anyone of claims 1-5, wherein at least one of said at least one vehicle comprises at least two nucleic acids.
- 20 7. A library according to claim 3, wherein said vehicle comprises nucleic acid derived from an adenovirus.
8. A library according to claim 7, wherein said nucleic acid derived from an adenovirus comprises nucleic acid encoding an adenovirus late protein or a functional part,  
25 derivative and/or analogue thereof.
9. A library according to anyone of claims 3, 7 or 8, wherein said nucleic acid derived from an adenovirus comprises nucleic acid encoding adenovirus E2A or a functional part, derivative and/or analogue thereof.
- 30 10. A library according to anyone of claims 3, 7-9, wherein said nucleic acid derived from an adenovirus comprises nucleic acid encoding at least one E4-region

protein or a functional part, derivative and/or analogue thereof.

11. A library according to anyone of claims 3, 7-10 wherein said nucleic acid derived from an adenovirus  
5 comprises nucleic acid encoding at least one E1-region protein or a functional part, derivative and/or analogue thereof.

12. A library according to anyone of claims 2-11 wherein said vehicle further comprises nucleic acid comprising an  
10 adeno-associated virus terminal repeat or a functional part, derivative and/or analogue thereof.

13. A library according to anyone of claims 3-12, wherein said viral element derived from an adenovirus comprises an adenovirus capsid or a functional part, derivative and/or  
15 analogue thereof.

14. A library according to anyone of claims 1-13, wherein said vehicle comprises adenovirus fiber proteins from at least two adenoviruses.

15. A method for determining at least one function of at least one nucleic acid present in a library according to anyone of claims 1-14, comprising transducing a multiplicity of cells with at least one vehicle comprising at least one nucleic acid from said library, culturing said cell while allowing for expression of said at least one nucleic acid and  
20 determining the expressed function.

16 A method according to claim 15, wherein said multiplicity of cells is divided over a number of compartments each comprising at least one vehicle comprising at least one nucleic acid from said library.

30 17 A method according to claim 15 or claim 16, further comprising selecting the vehicle comprising a desired function.

18 A method for obtaining an expressible nucleic acid having a desired function when expressed in a cell comprising  
35 determining at least one function of at least one nucleic acid present in a library according to anyone of claims 1-14,

said method comprising transducing a multiplicity of cells with at least one vehicle comprising at least one nucleic acid from said library, culturing said cell while allowing for expression of said at least one nucleic acid and  
5 determining the expressed function.

19 A method for producing a library comprising a multiplicity of compartments each comprising at least one nucleic acid delivery vehicle each comprising at least one nucleic acid, said method comprising recombining vehicle  
10 nucleic acid with said at least one nucleic acid, thereby producing a vehicle capable of delivering said at least one nucleic acid to a cell in an expressible manner.

20. A method according to claim 19, wherein said recombining comprises homologous recombination between at  
15 least partially overlapping sequences in vehicle nucleic acid and said at least one nucleic acid.

21. A method according to claim 19 or claim 20, wherein said vehicle nucleic acid and/or said at least one nucleic acid comprises adenovirus nucleic acid or a functional part,  
20 derivative and/or analogue thereof.

22. A method according to claim 21, wherein said adenovirus nucleic acid comprises a host range mutation that enables adenovirus to replicate in non human primate cells.

23. A library obtainable by a method according to anyone  
25 of claims 19-22.

24 Use of a library according to claim 23 in a method according to anyone of claims 15-18.

25. A method for amplifying a vehicle present in a library according to anyone of claims 3-14, or 23, comprising  
30 providing a cell with said vehicle, culturing said cell allowing the amplification of said vehicle and harvesting vehicles amplified by said cell.

26. A method according to claim 25, wherein said cell comprises nucleic acid encoding an adenovirus E1-region  
35 protein.

27. A method according to claim 26, wherein said cell is a PER.C6 cell (ECACC deposit number 96022940) or a functional derivative and/or analogue thereof.

28. A method according to claim 26 or claim 27, wherein  
5 said cell further comprises nucleic acid encoding adenovirus E2A and/or an adenovirus E4-region protein or a functional part, derivative and/or analogue thereof.

29. A method according to anyone of claims 26-28, wherein  
10 vehicle nucleic acid does not comprise sequence overlap with other nucleic acid present in said cell leading to the formation of vehicle nucleic acid capable of replicating in the absence of E1-region encoded proteins.

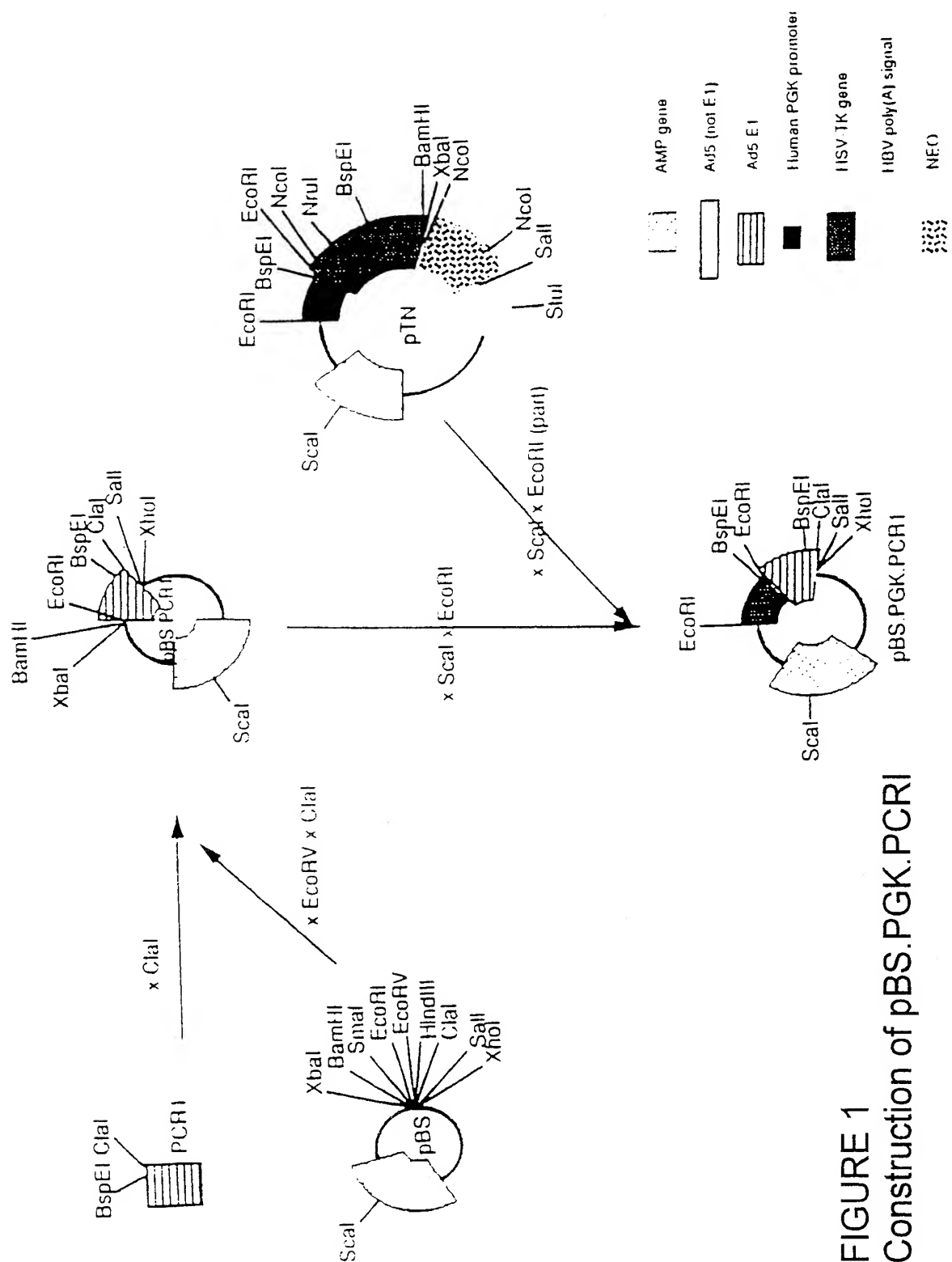
30. A library according to anyone of claims 1-14 or  
method according to anyone of claims 15-22, 25-29, wherein  
15 said multiplicity of compartments comprises a multiwell format.

31. A library according to anyone of claims 1-14 or a  
method according to anyone of claims 15-22, 25-30, wherein  
said at least one nucleic acid encodes a product of unknown  
20 function.

32. A library according to anyone of claims 1-14 or a  
method according to anyone of claims 15-22, 25-32, wherein  
said library is used or said method is performed in an at  
least substantially automated setting.

25 33. A multiplicity of cells comprising a library  
according to anyone of claims 1-14 or 23.

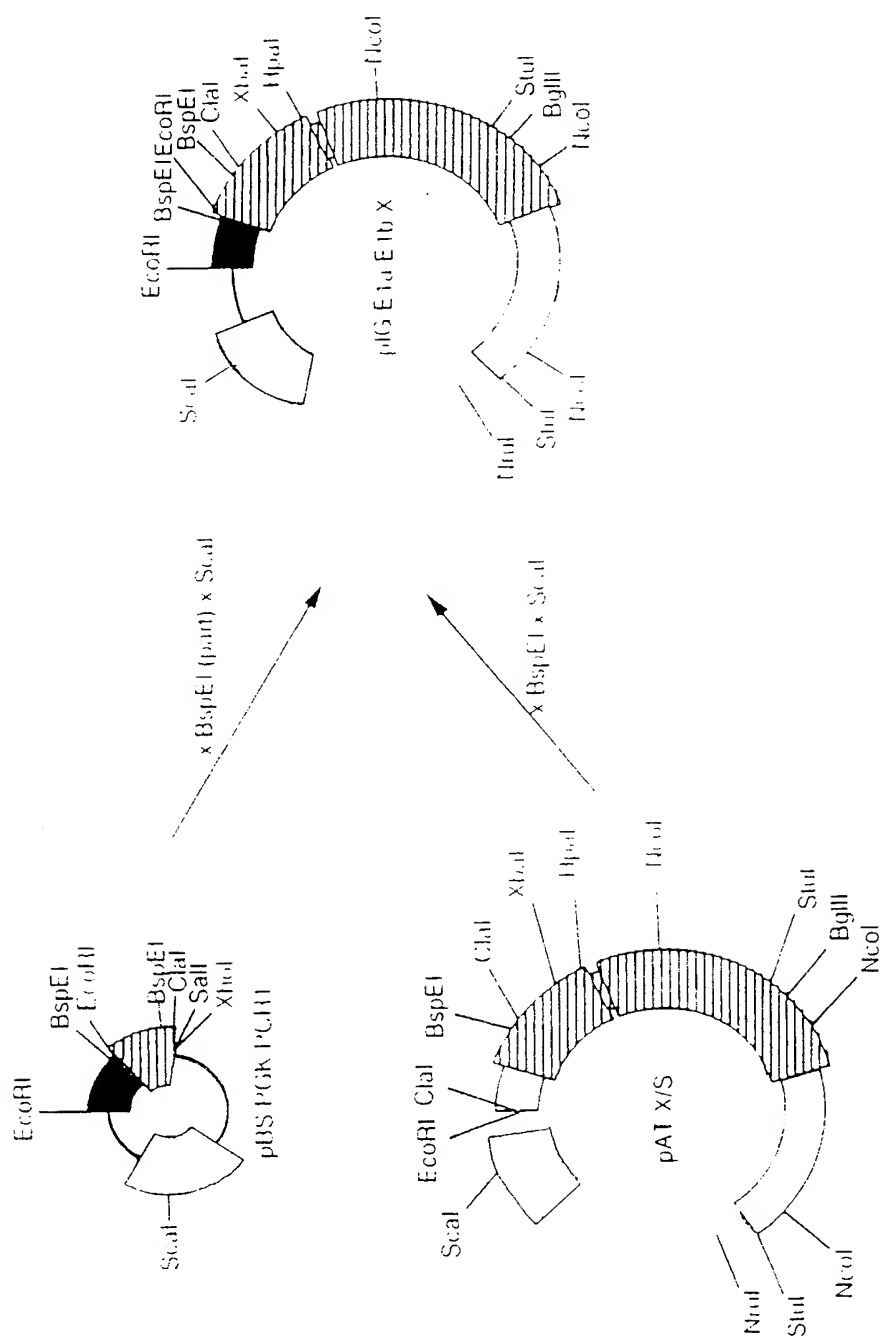
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**FIGURE 1**  
Construction of pBS.PGK.PCRI



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**FIGURE 2**  
Construction of plG.E1a.E1b.X

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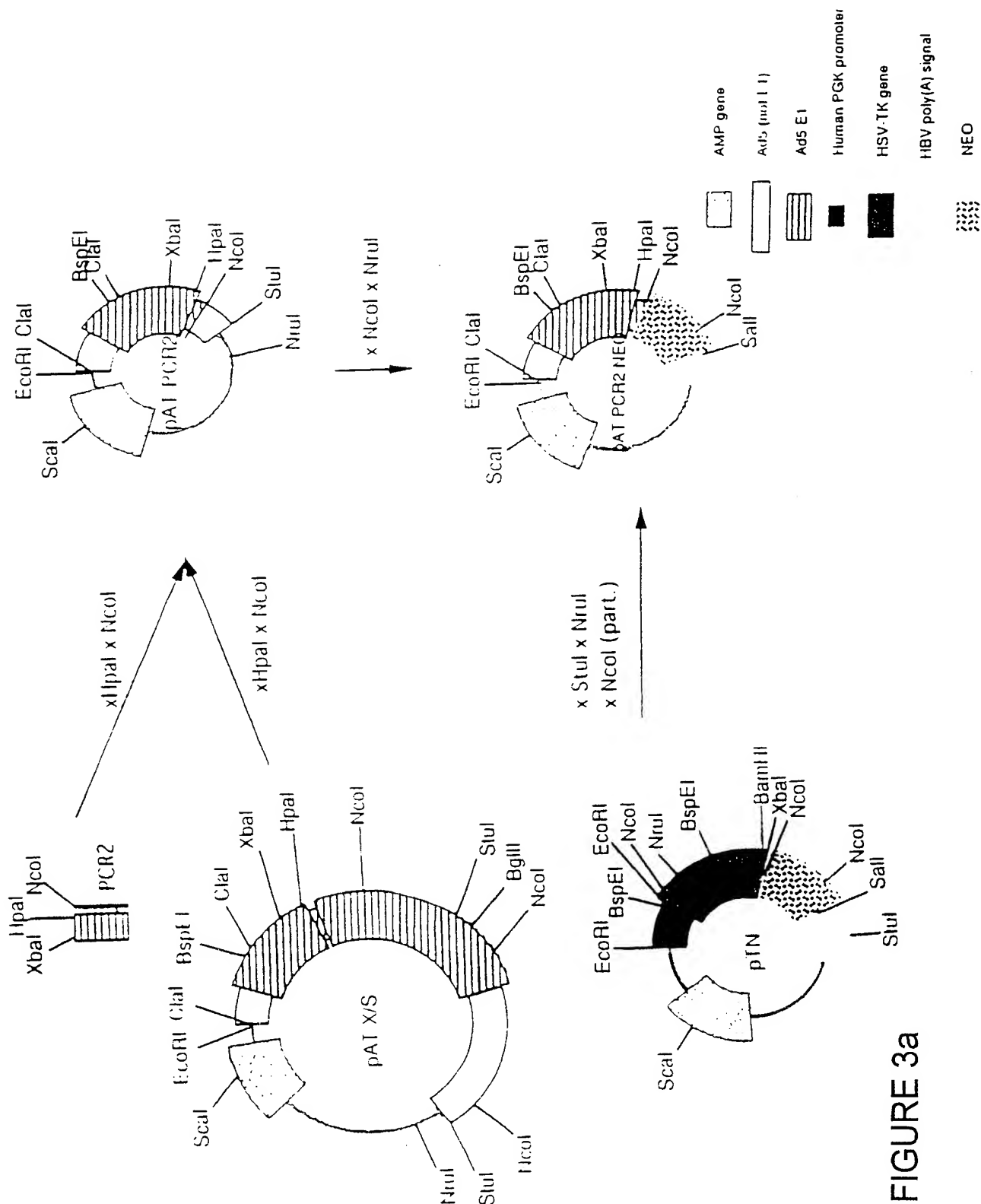
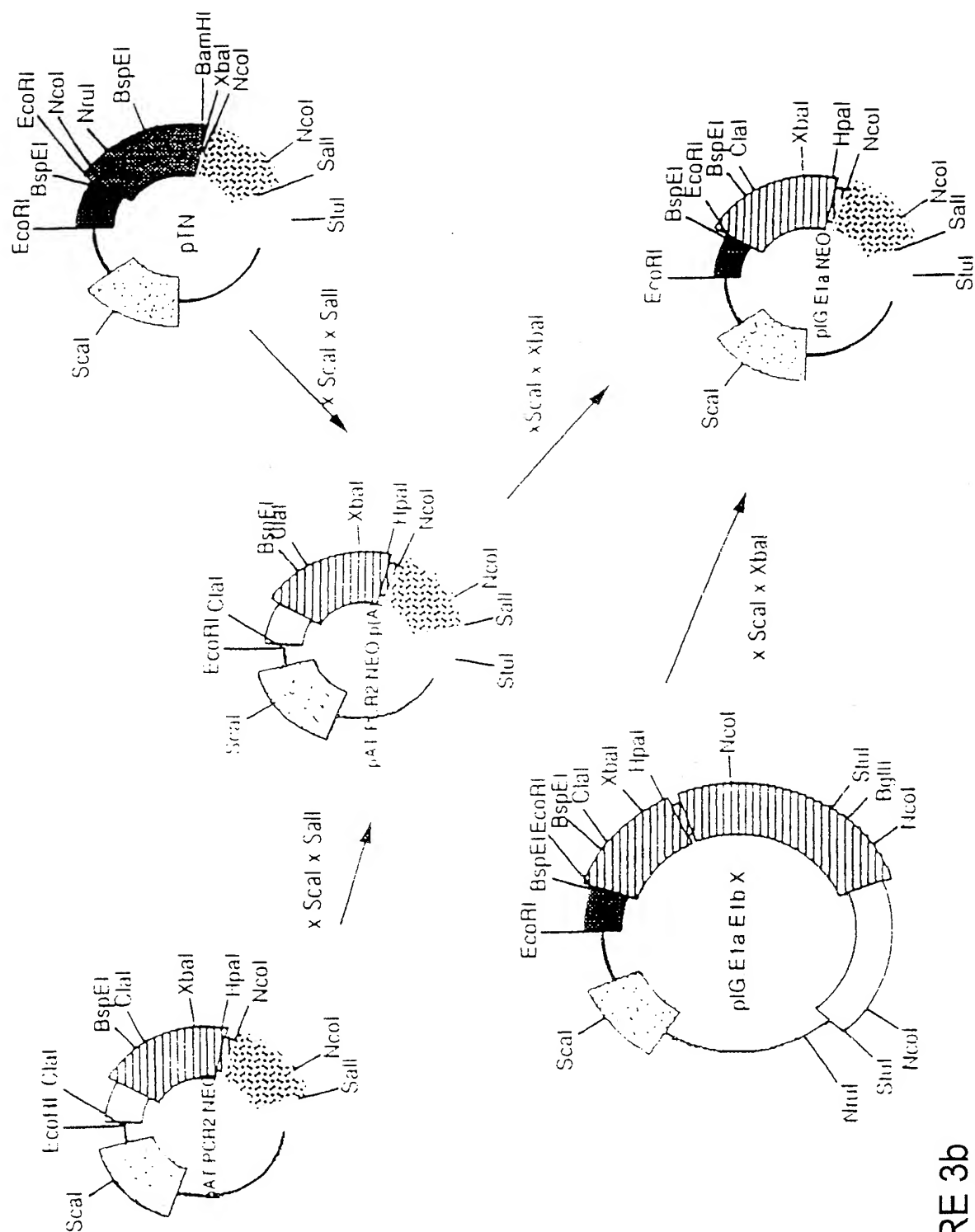


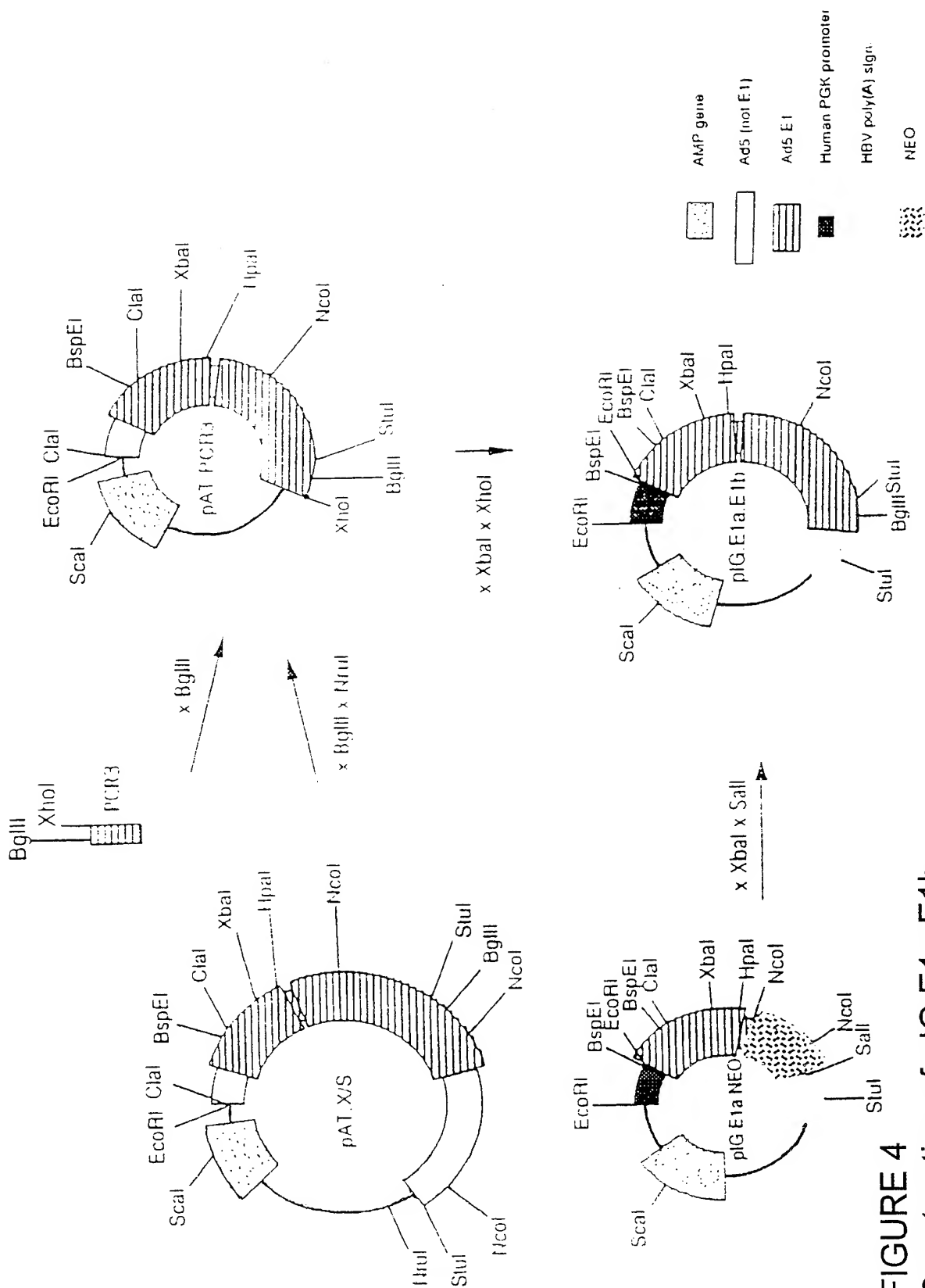
FIGURE 3a

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**FIGURE 3b**  
Construction of plG.E1a.NEO

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**FIGURE 4**  
Construction of plG.E1a.E1b

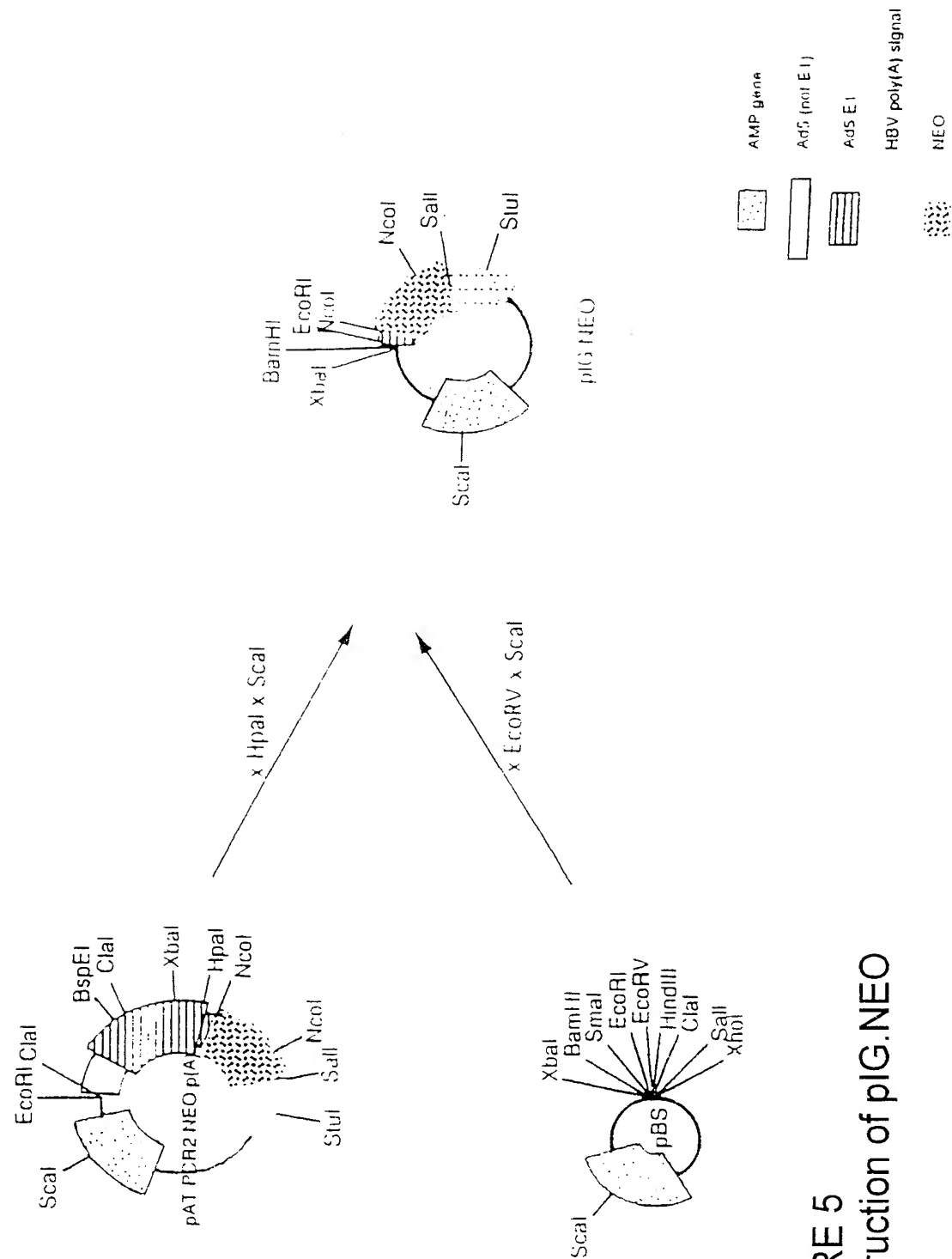
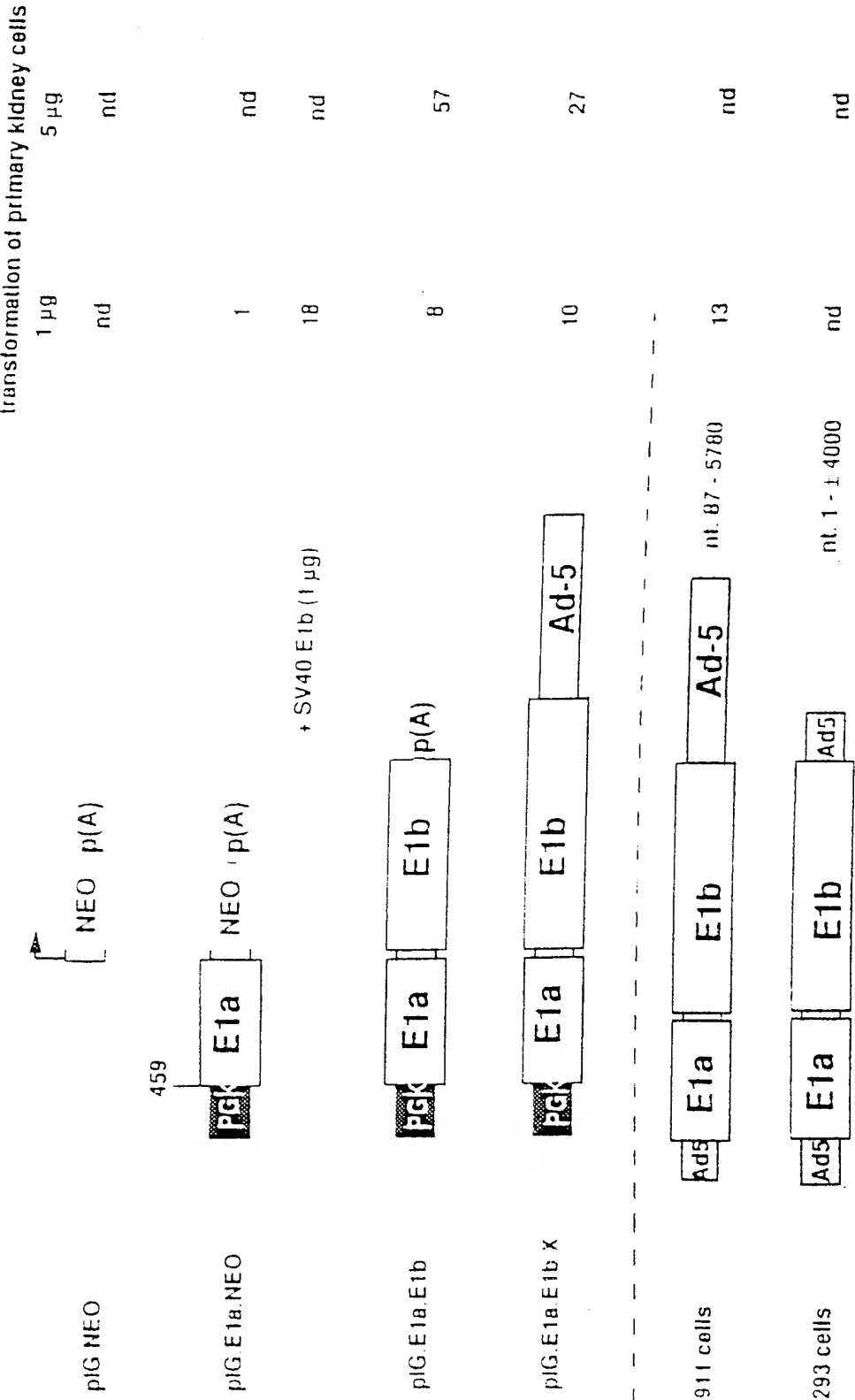


FIGURE 5  
Construction of pIG.NEO



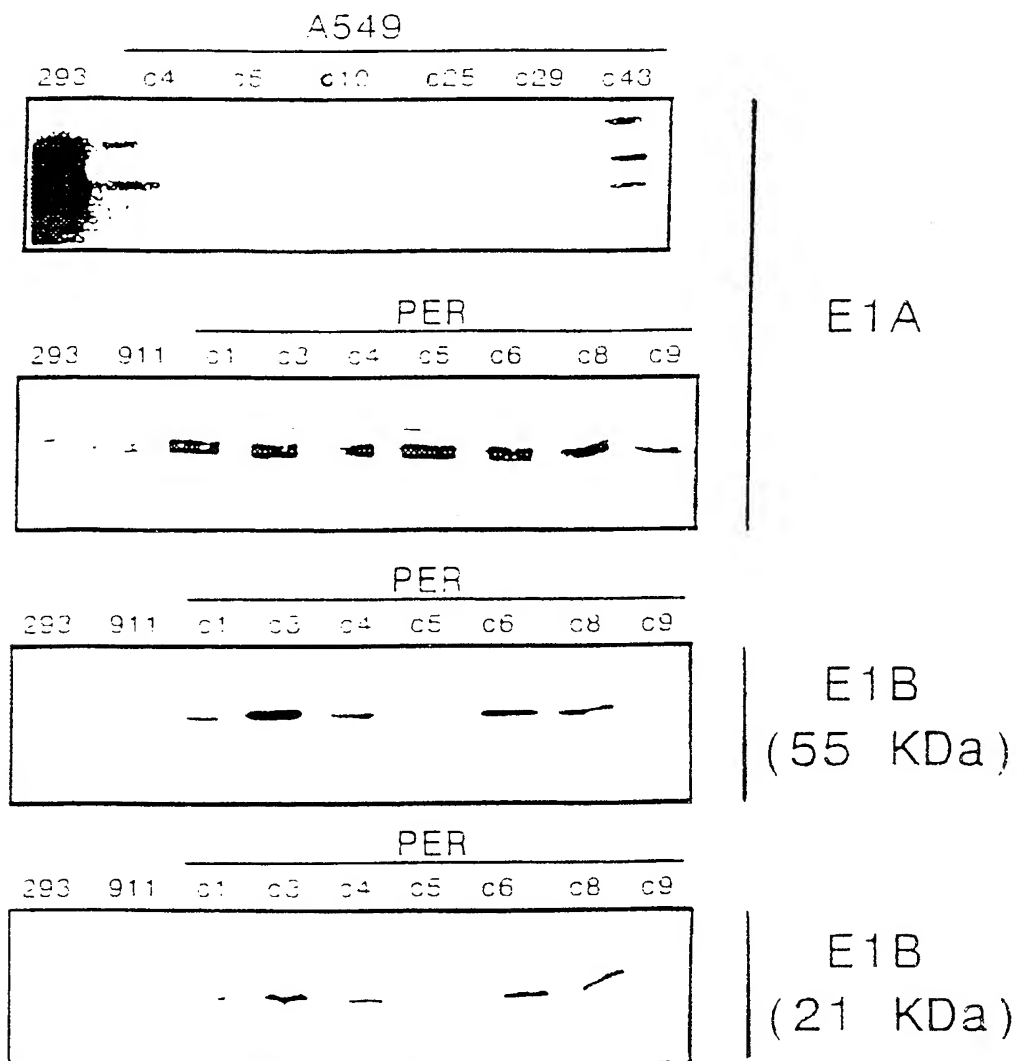
\* average of 5 plates 21 days after transfection

**FIGURE 6**  
Overview of available adenovirus packaging constructs and assessment of their capacity to transform primary kidney cells

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## FIGURE 7

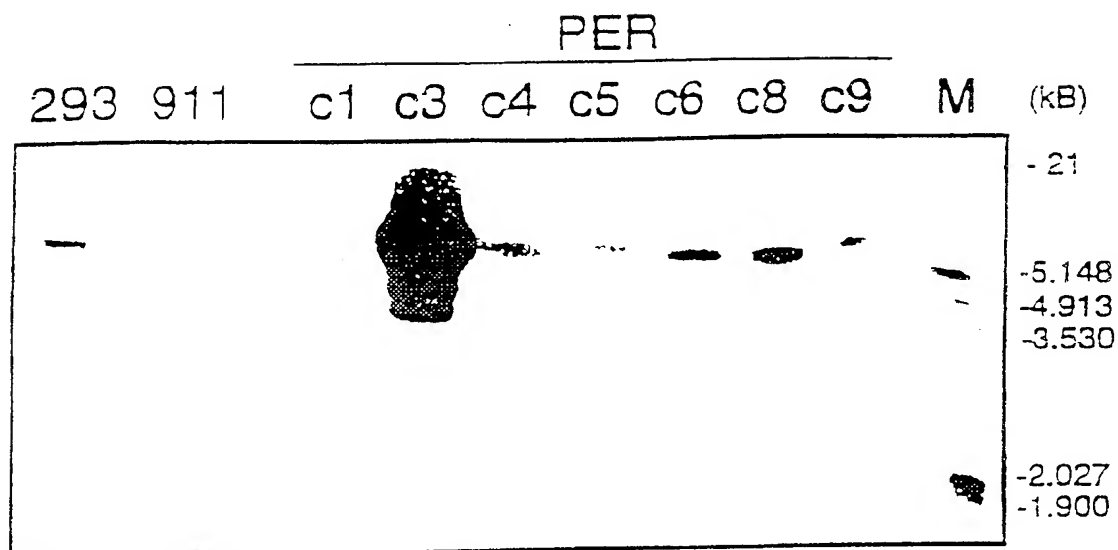
Western blotting analysis of A549 clones transfected with pIG.E1A.NEO and PER clones (HER cells transfected with pIG.E1A.E1B)



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## FIGURE 8

Southern blot analyses of 293, 911 and PER cell lines

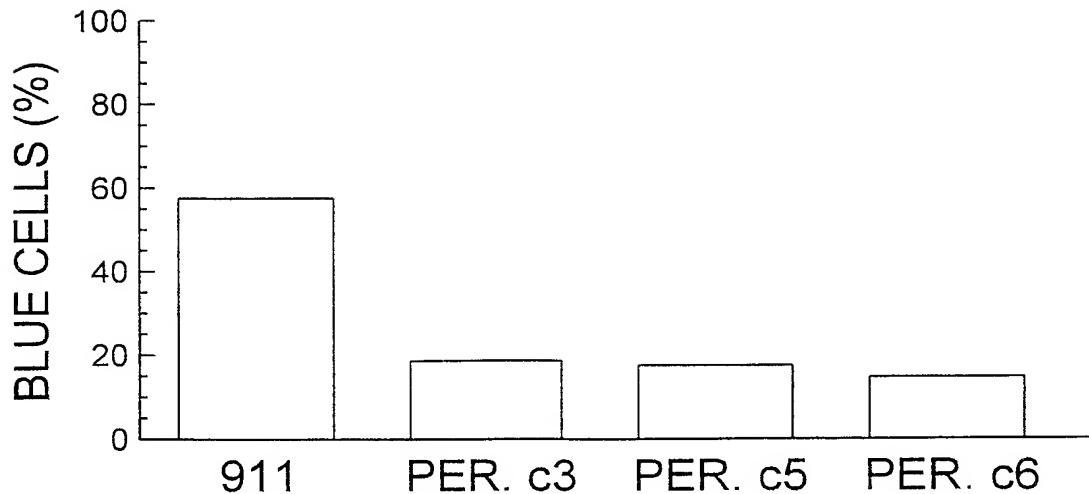




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## FIGURE 9

Transfection efficiency of PER.C3, PER.C5, PER.C6 and 911 cells. Cells were cultured in 6-well plates and transfected (n=2) with 5  $\mu$ g pRSV.lacZ by calcium-phosphate co-precipitation. Forty-eight hours later the cells were stained with X-GAL. The mean percentage of blue cells is shown.



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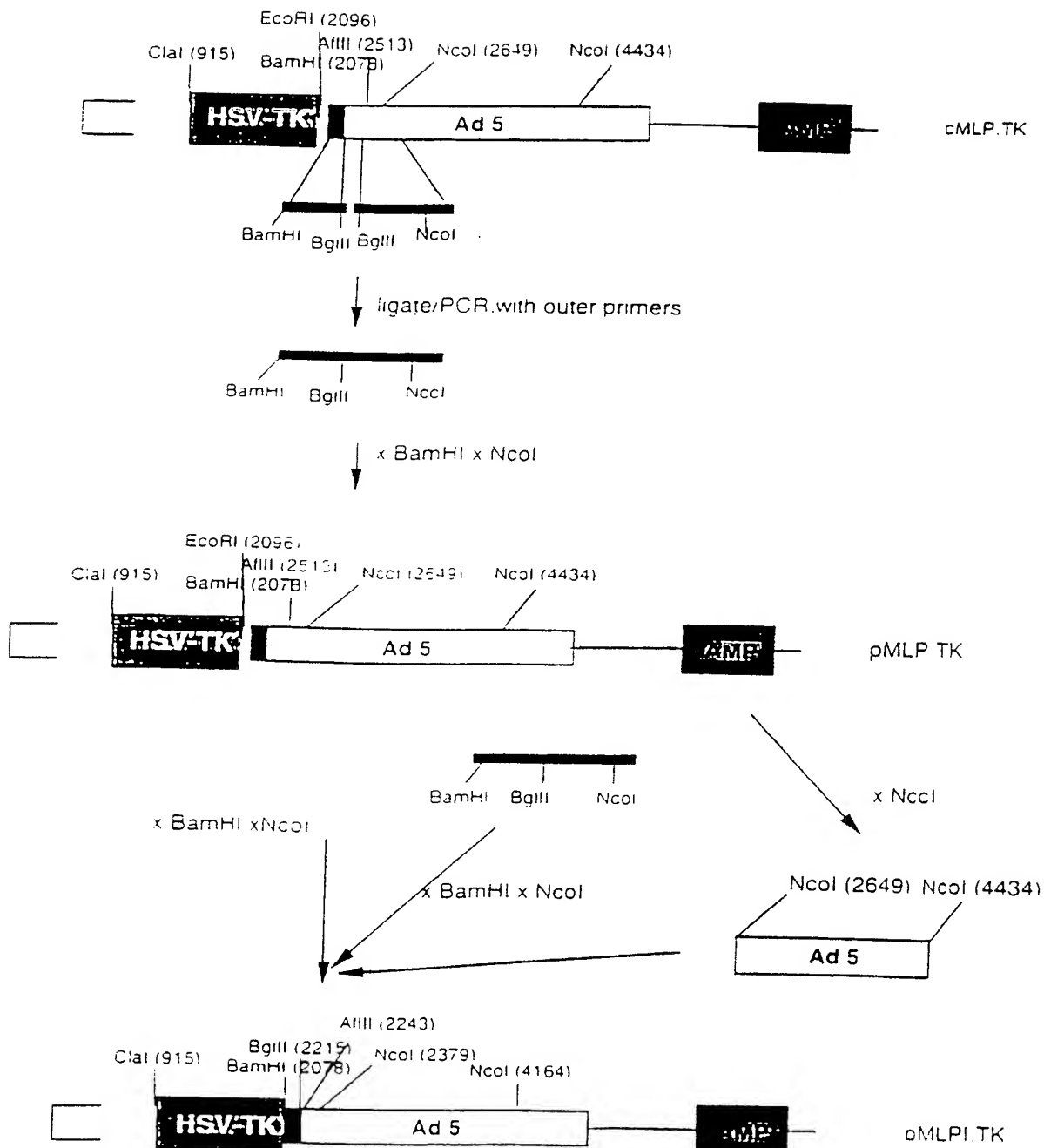


FIGURE 10  
Construction of pMLPI.TK from pMLP.TK

New recombinant adenoviruses and packaging constructs without sequence overlap

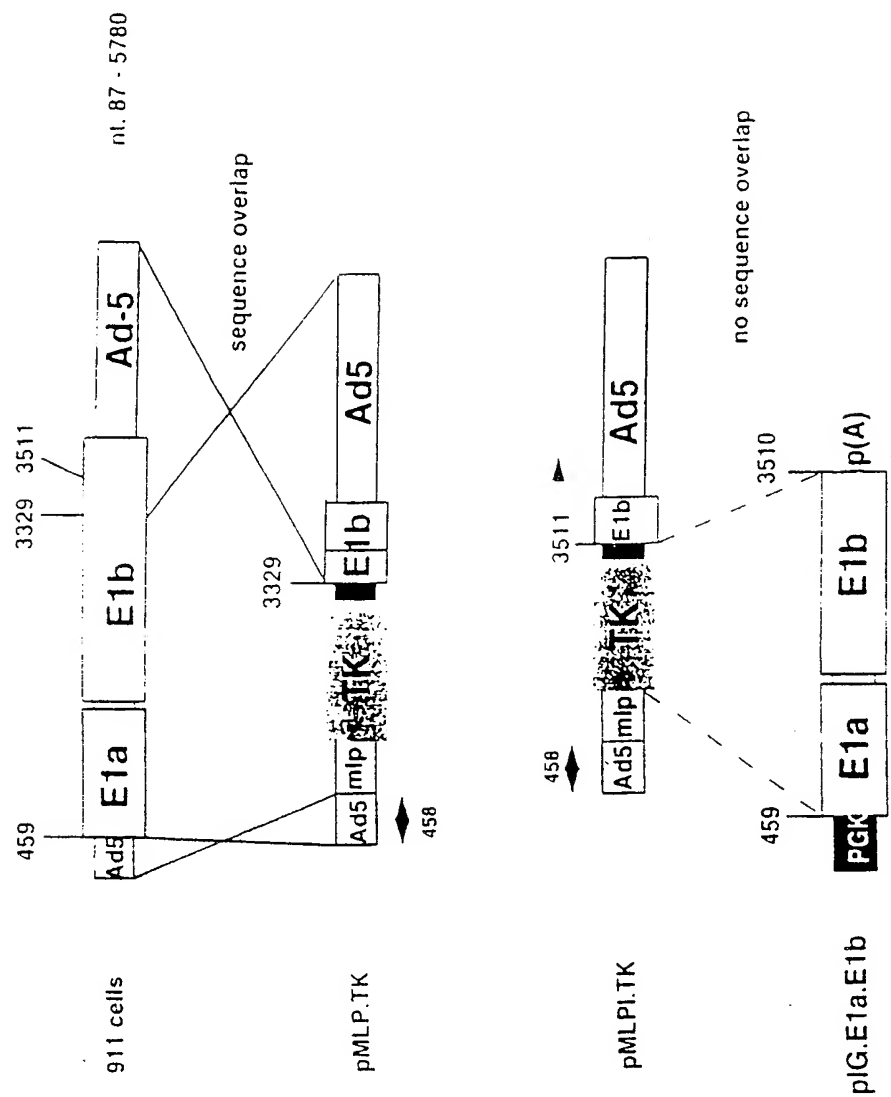


FIGURE 11a  
Packaging system based on primary cells

New recombinant adenoviruses and packaging constructs without sequence overlap

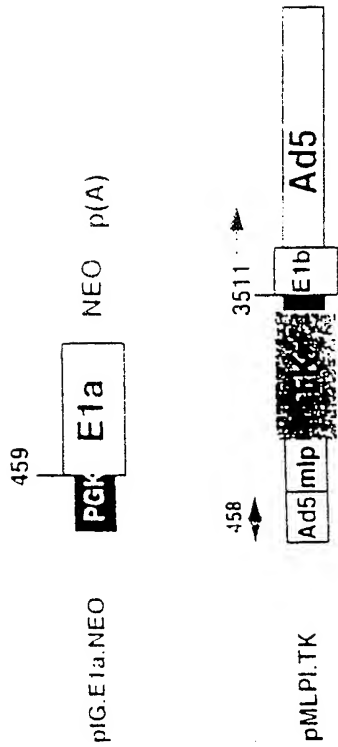


FIGURE 11.b  
Packaging system based on established cell lines: transfection with E1a and selection with G418

# Generation of recombinant adenovirus

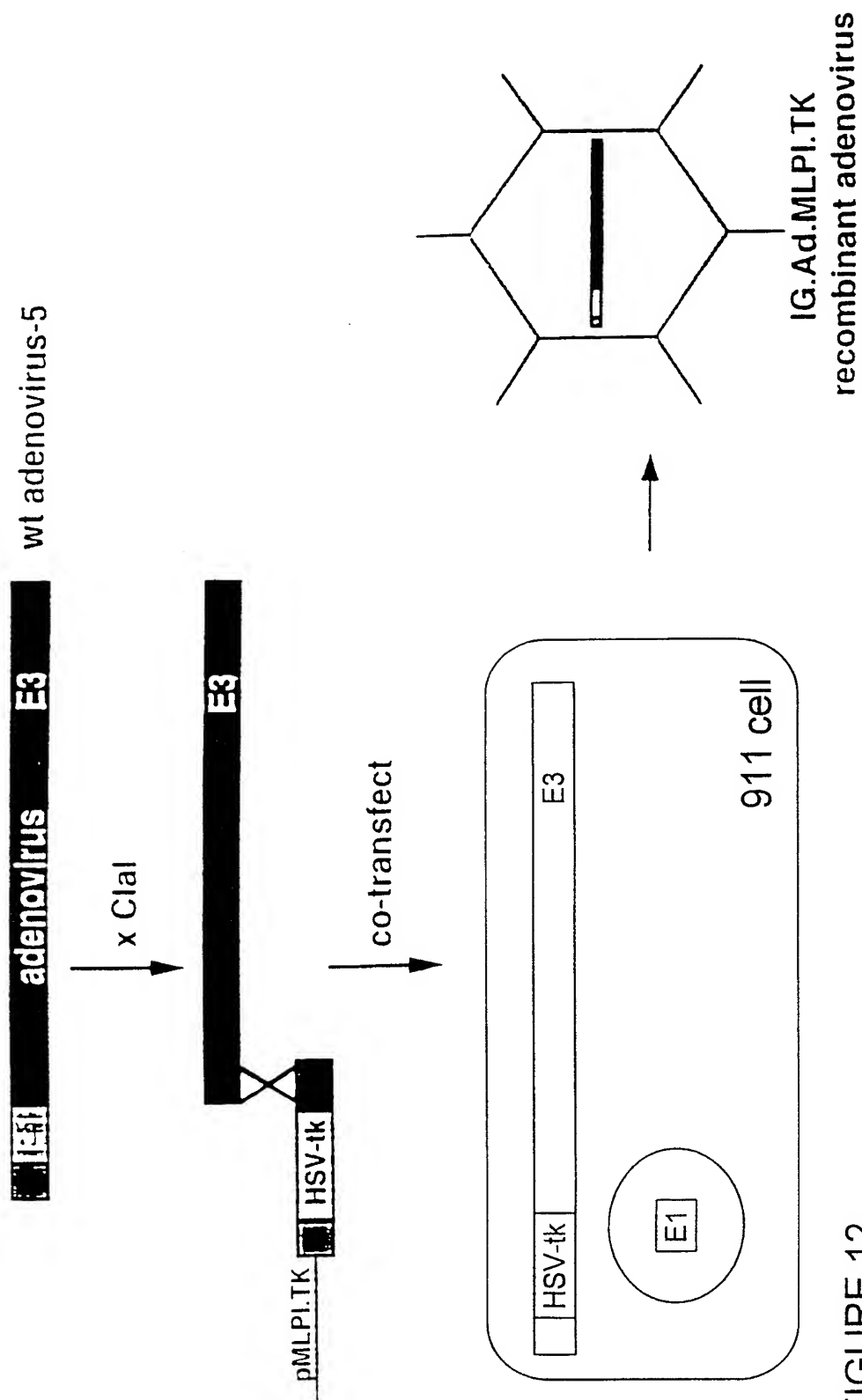
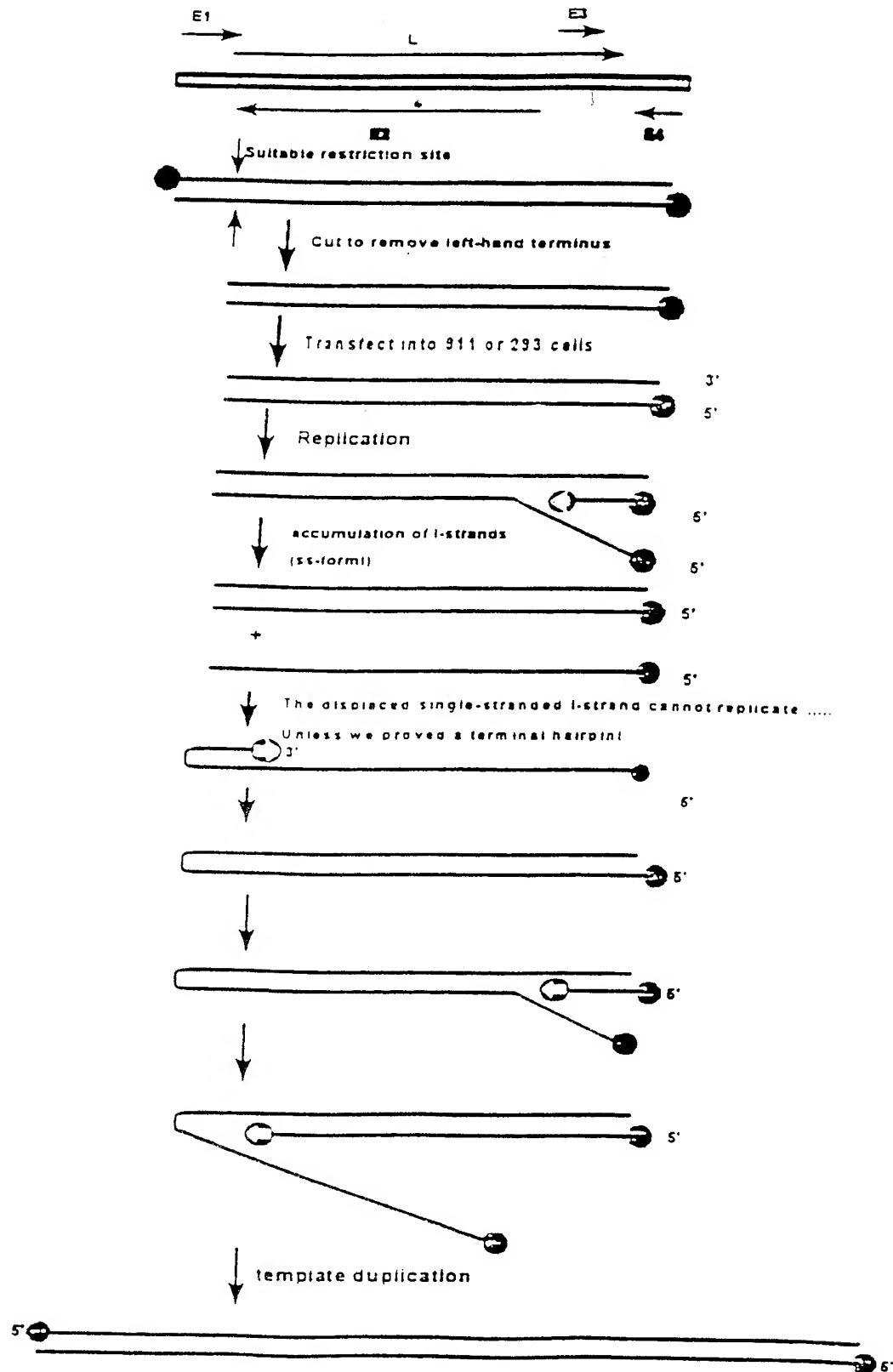


FIGURE 12

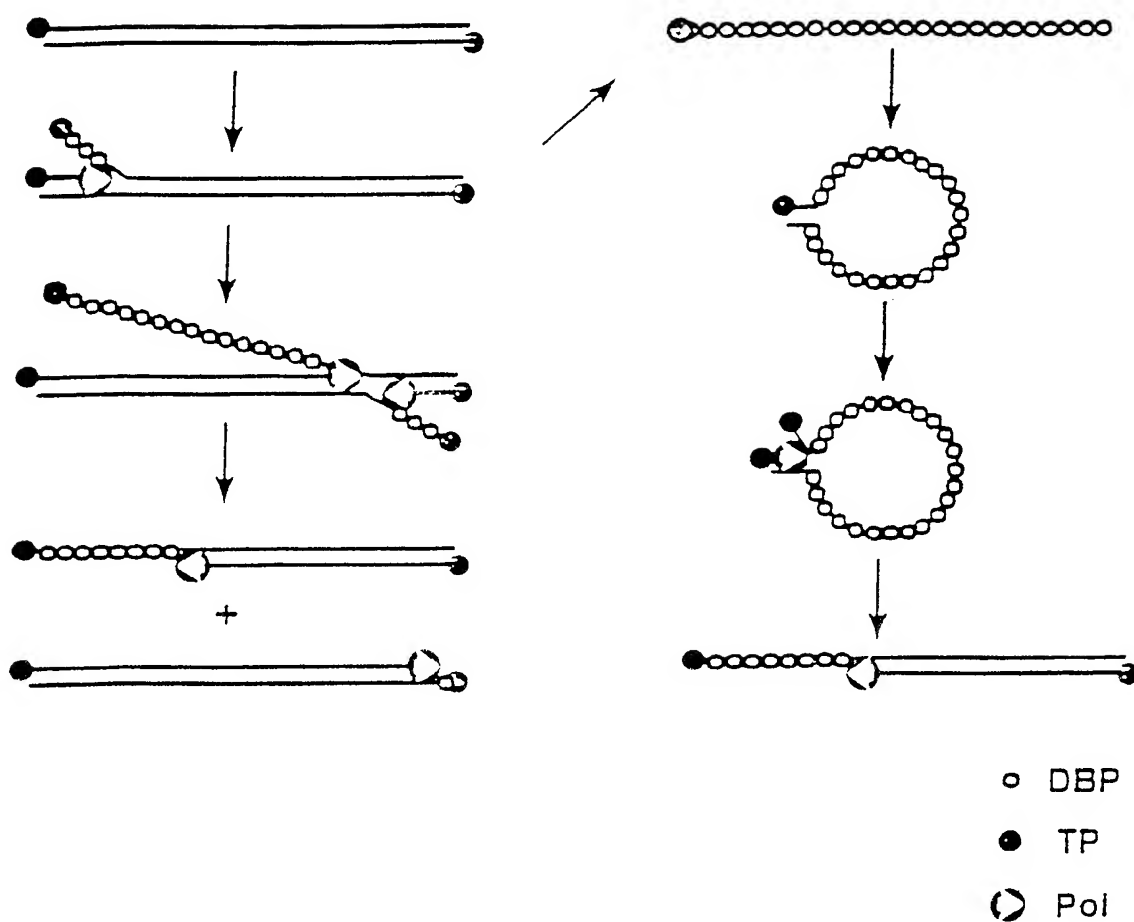
15/60  
FIGURE 13



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FIGURE 14

## Replication of Adenovirus



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## FIGURE 15

The potential hairpin conformation of a single-stranded DNA molecule that contains the HP/asp sequences used in these studies. Restriction with the restriction endonuclease Asp718I of plasmid pICLha., containing the annealed oligonucleotide pair HP/asp1 and HP/asp2 will yield a linear double-stranded DNA fragment. In cells in which the required adenovirus genes are present, replication can initiate at the terminus that contains the ITR sequence. During the chain elongation, the one of the strands will be displaced. The terminus of the single-stranded displaced-strand molecule can adopt the conformation depicted above. In this conformation the free 3'-terminus can serve as a primer for the cellular and/or adenovirus DNA polymerase, resulting in conversion of the displaced strand in a double-stranded form.

```

5'-GTACACTGACCTAGTCCCGCCCGGGCA
   |||||
3'-GATCAGCGGGGGCCCGA

```



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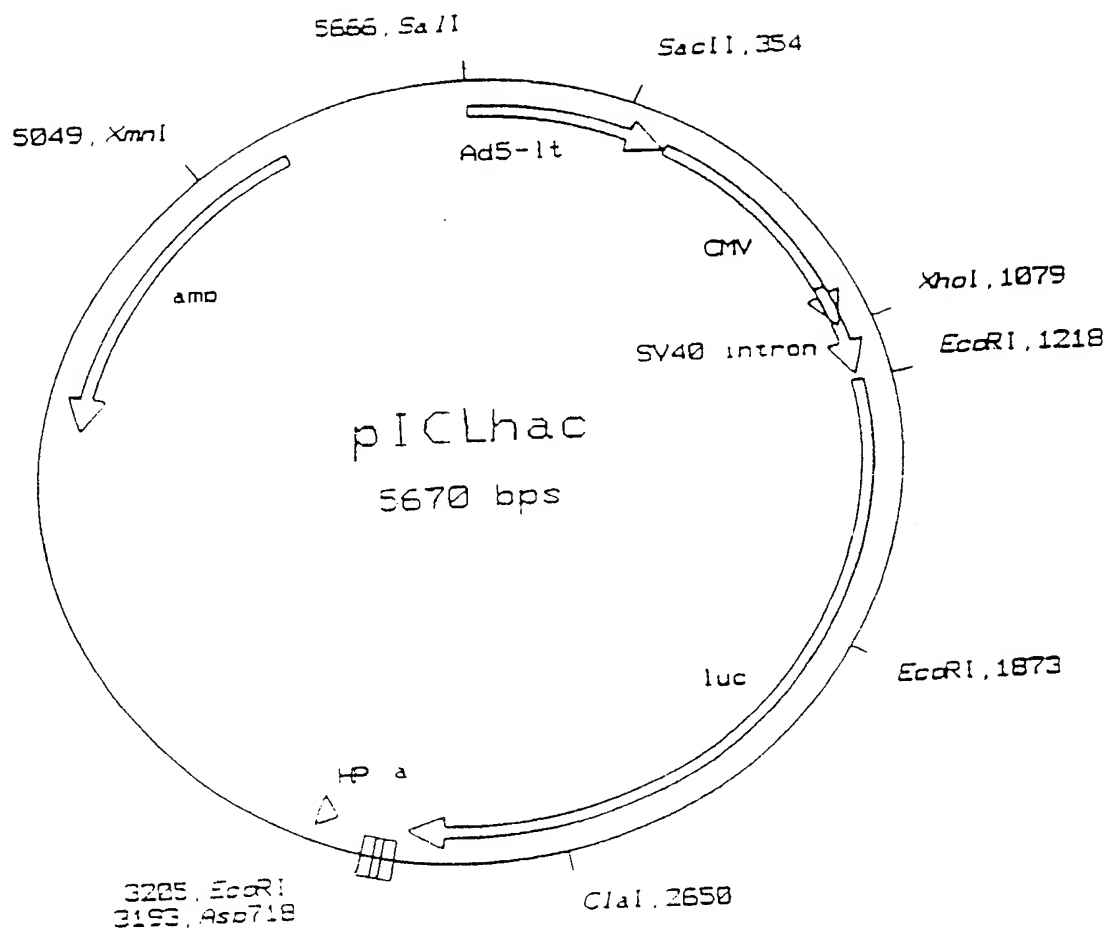
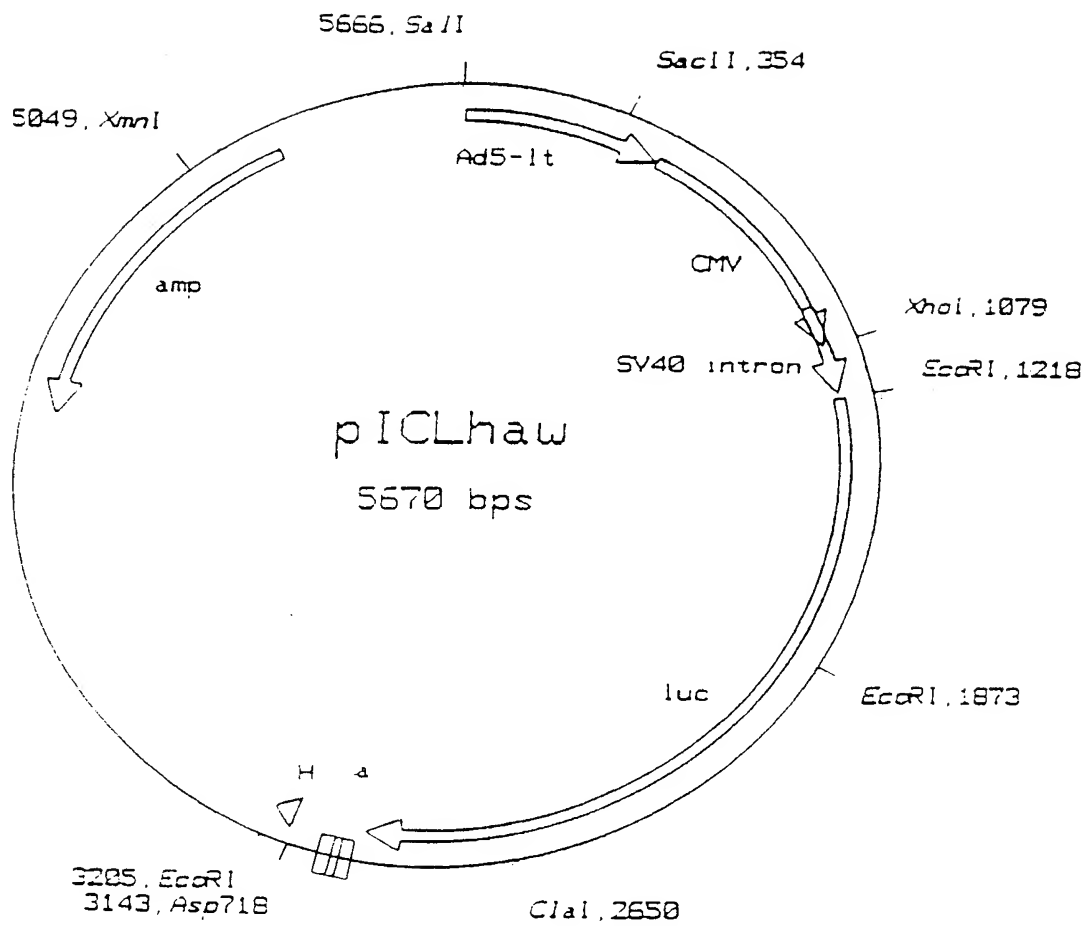


FIGURE 16

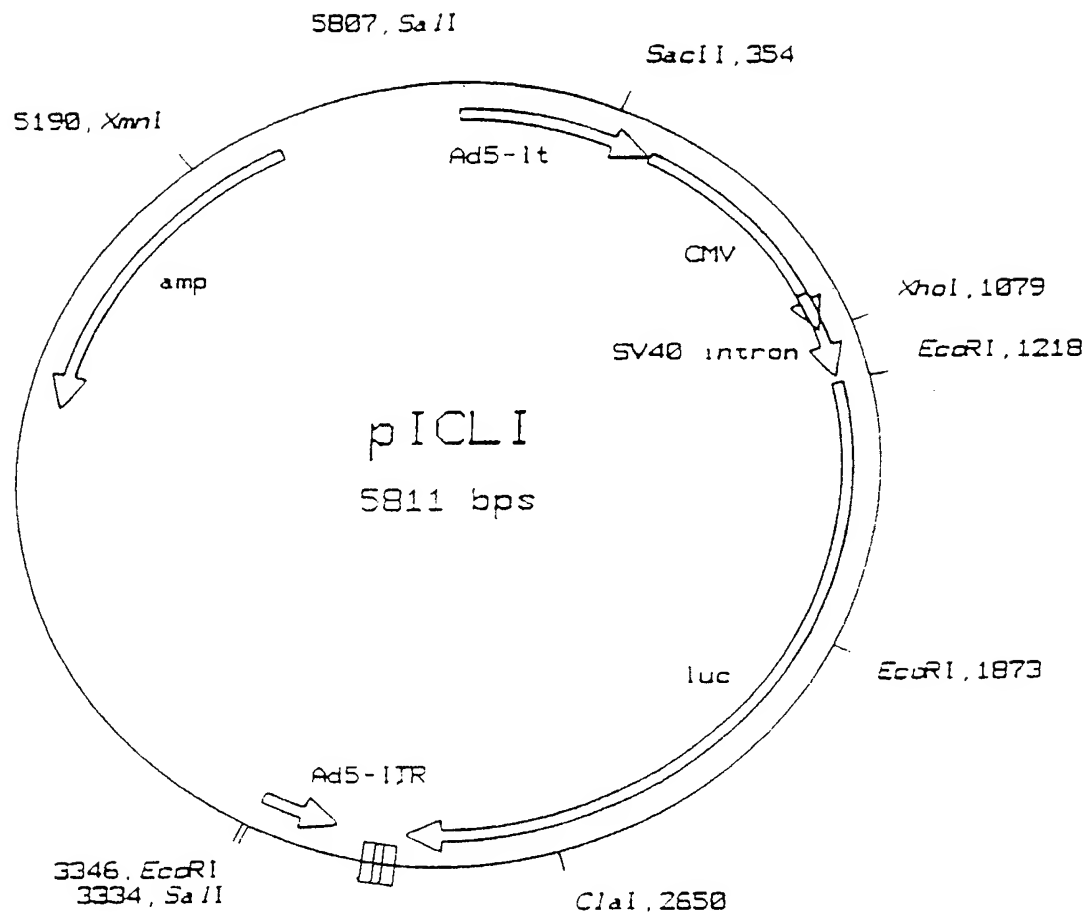
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FIGURE 17



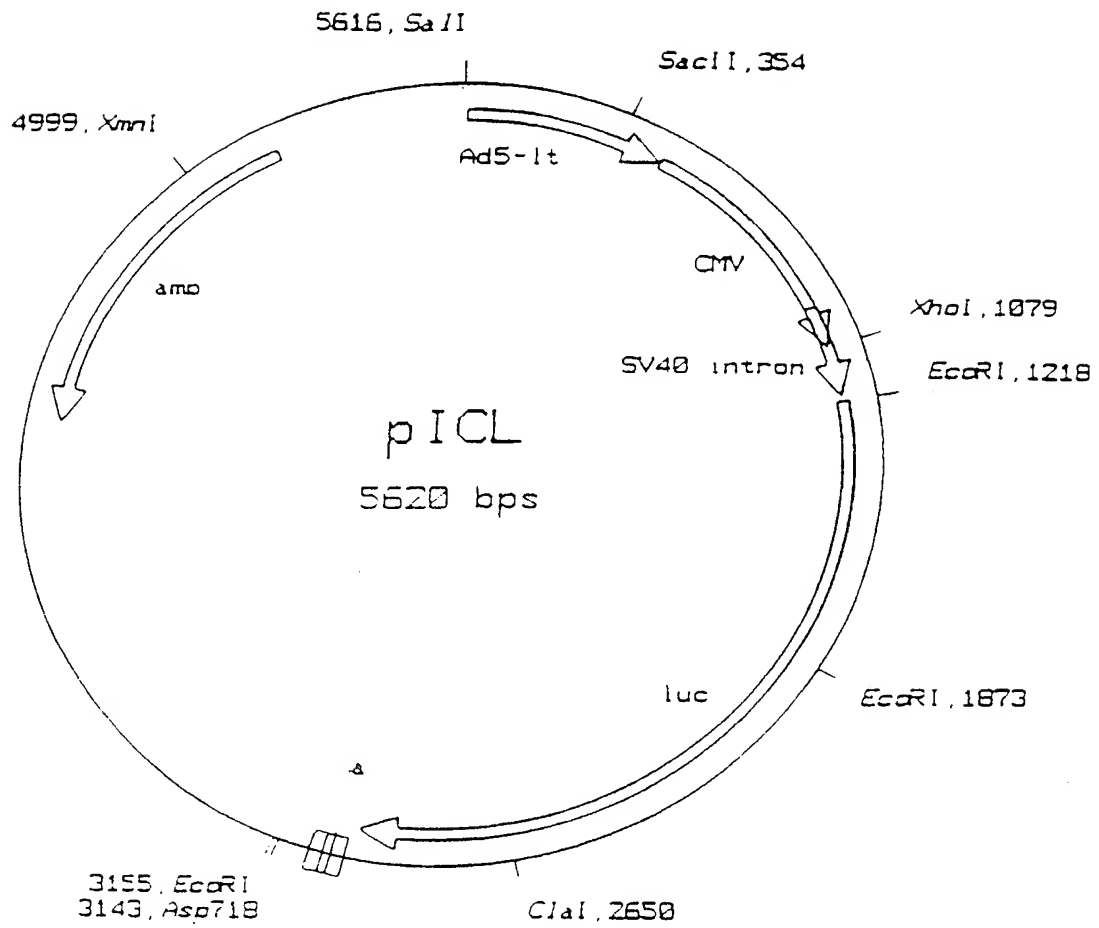
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FIGURE 18



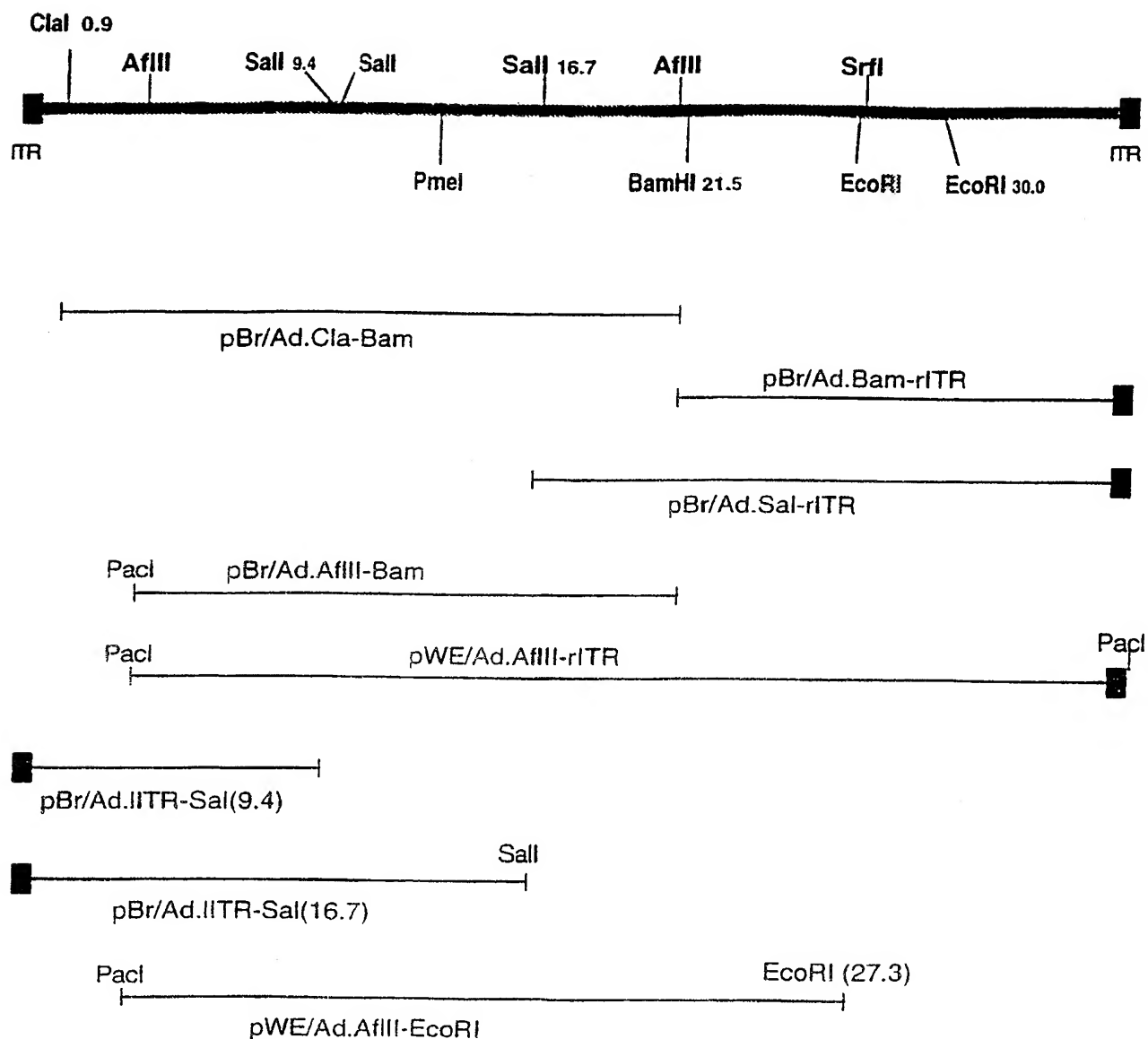
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FIGURE 19



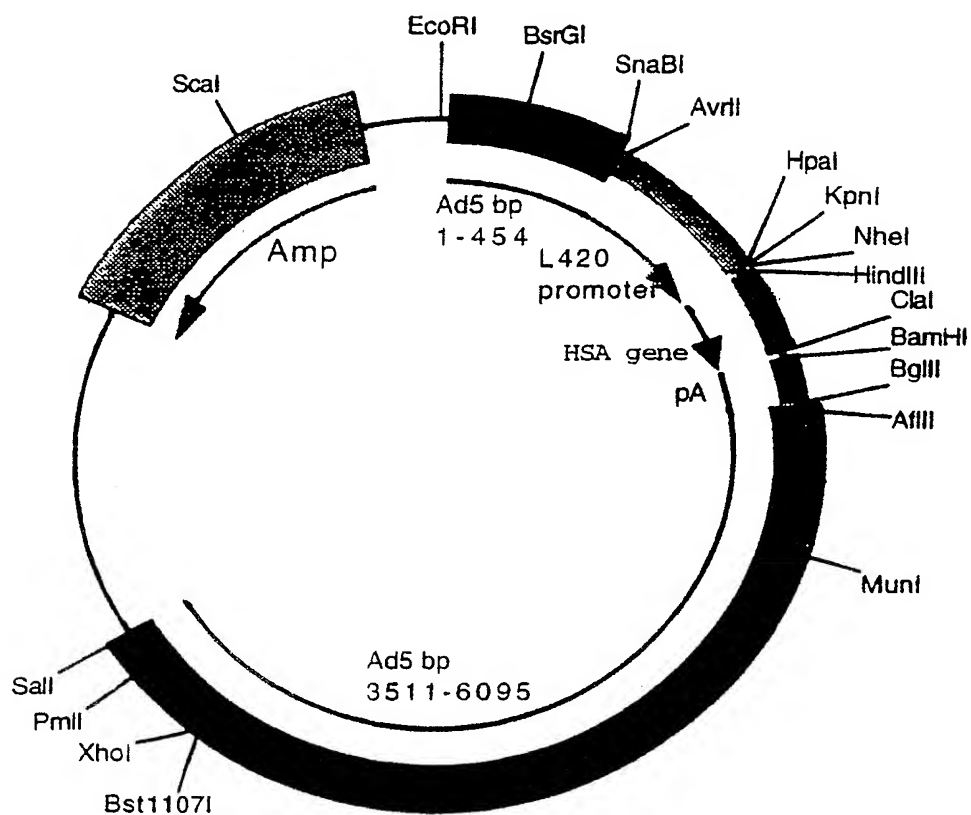
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FIGURE 20: Cloned adenovirus fragments



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FIGURE 21: Adapter plasmid pAd5/L420-HSA



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FIGURE 22: Adapter plasmid pAd5/CLIP

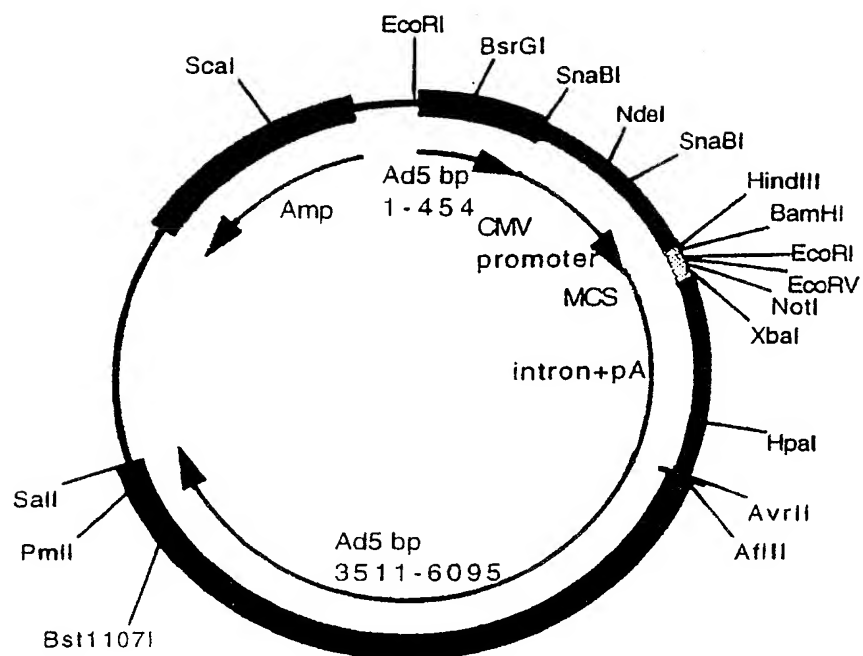
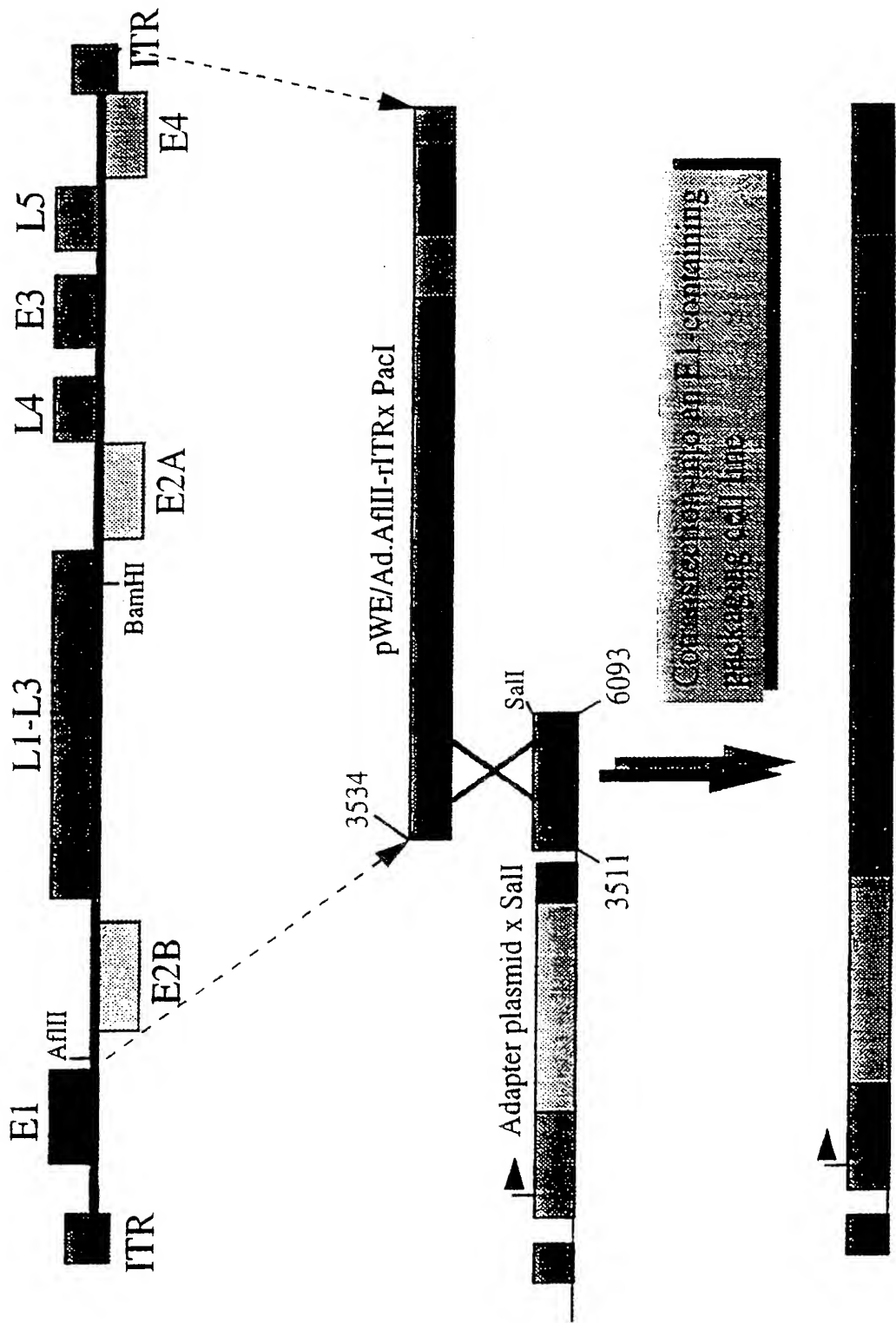


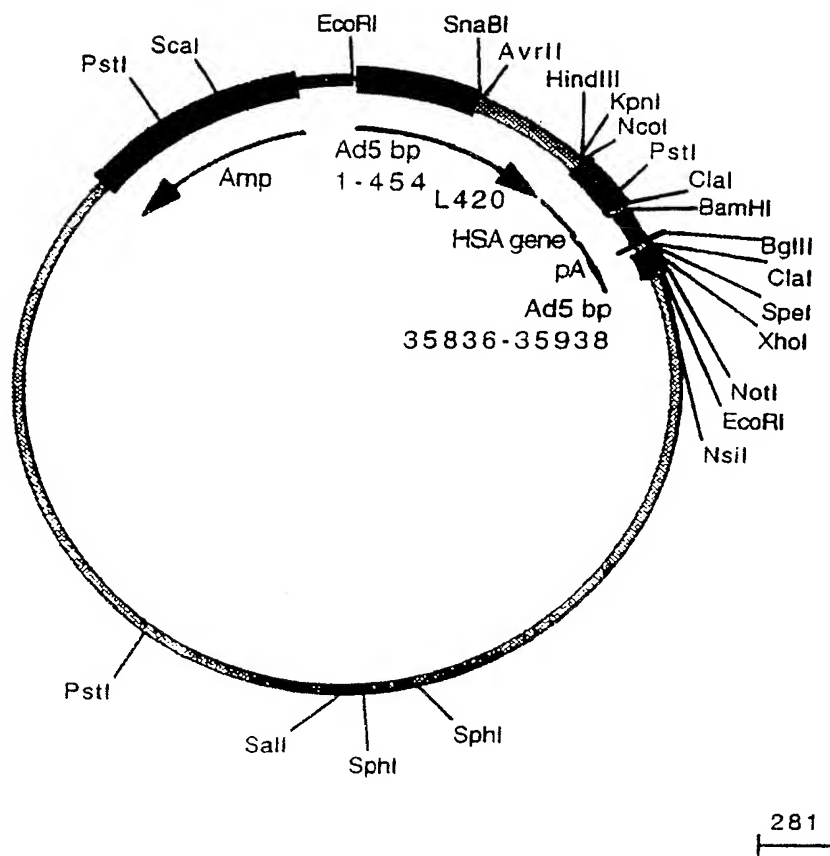
FIGURE 23: Generation of recombinant adenoviruses





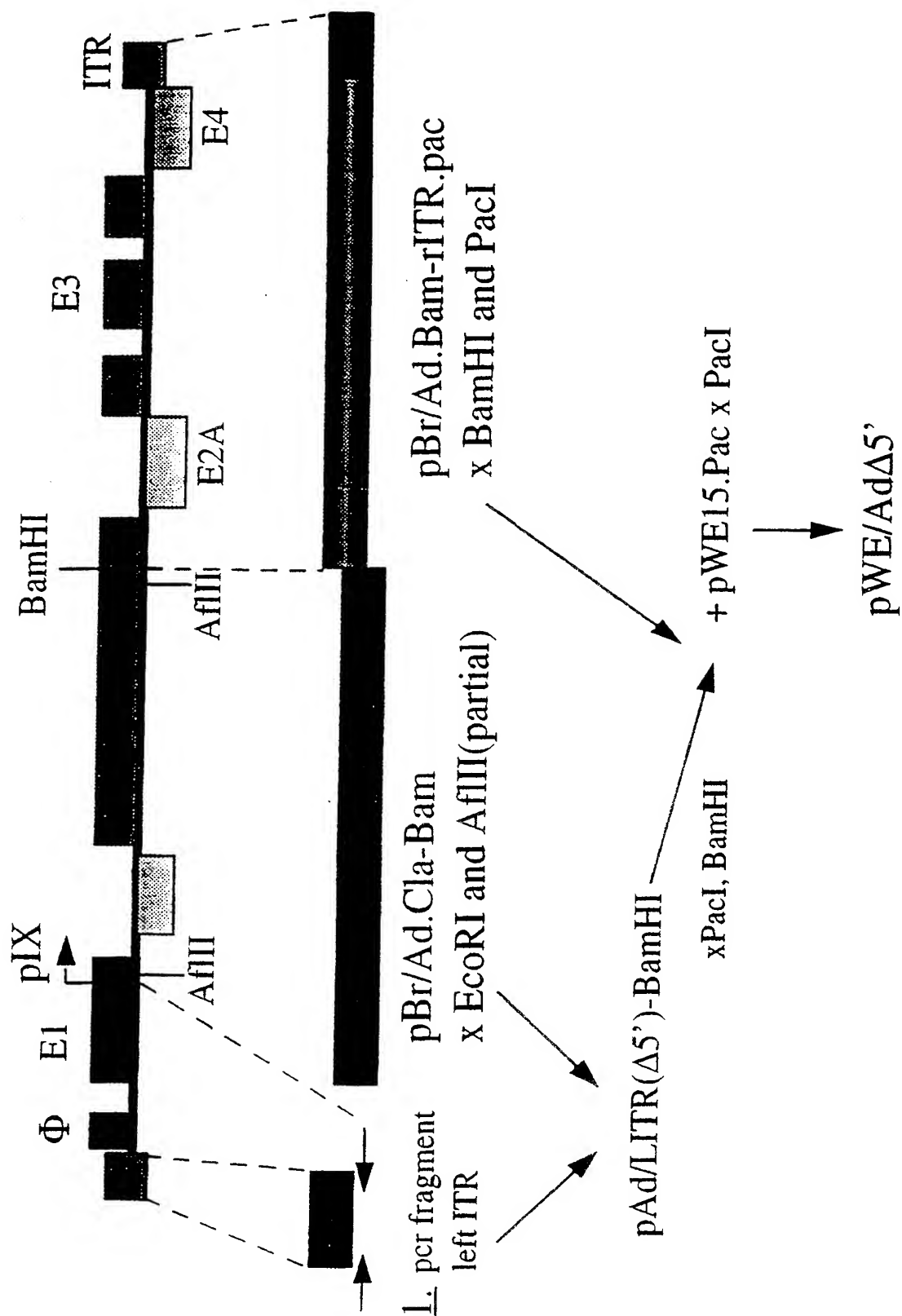
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FIGURE 24: Minimal adenovirus vector pMV/L420H



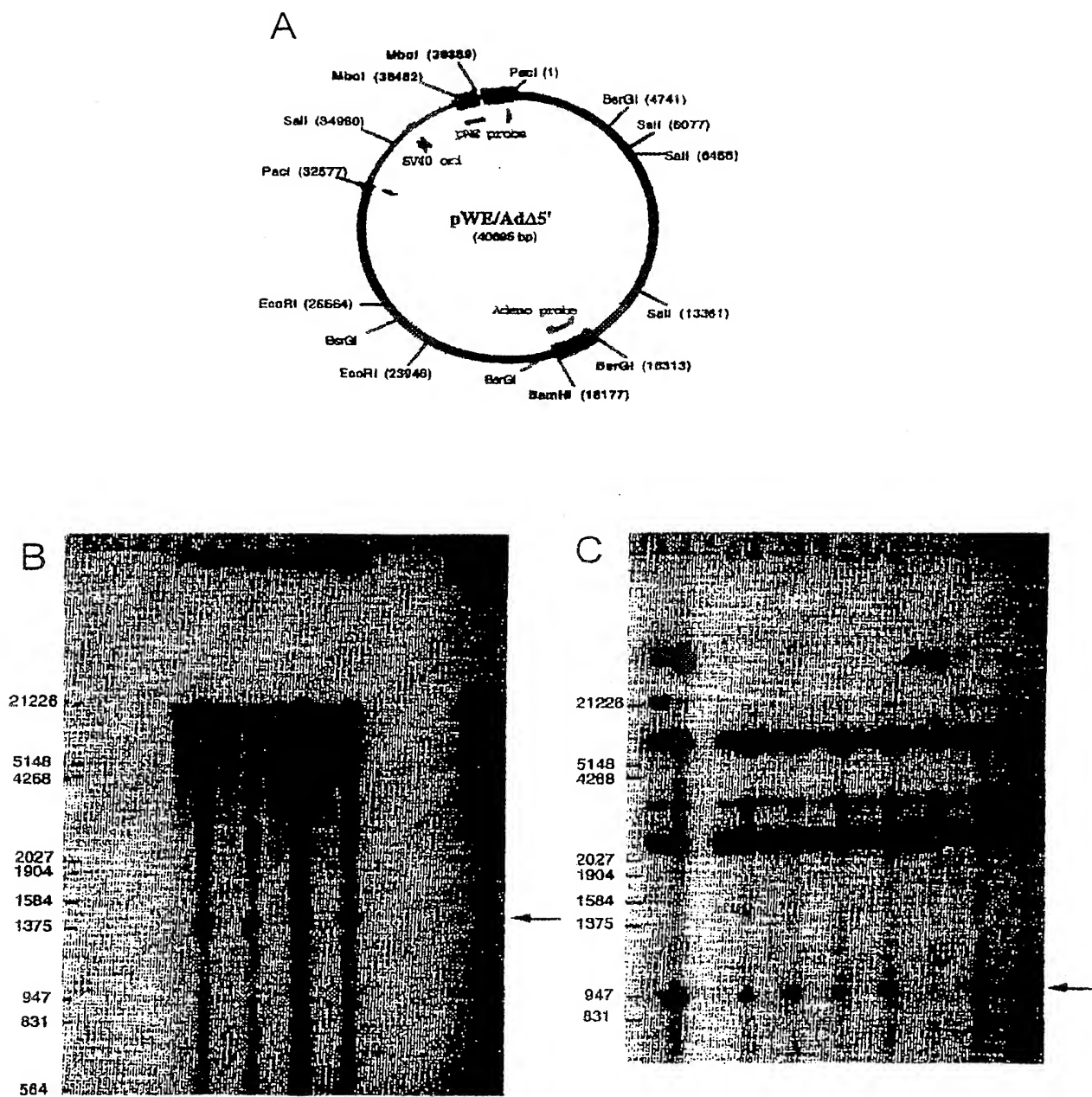
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FIGURE 25: Construction of pWE/AdΔ5'



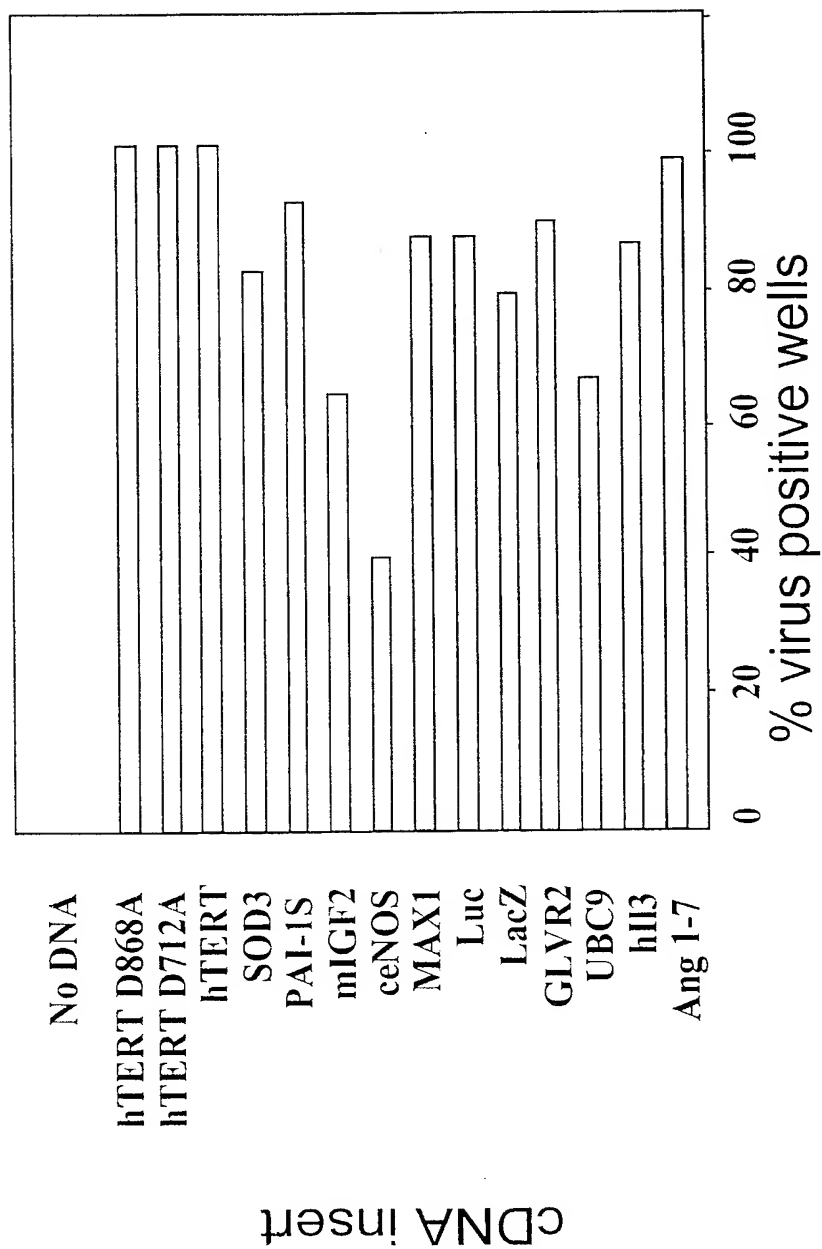
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FIGURE 26



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FIGURE 27



Average percentage CPE efficiency: 86 %

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Gene	Insert kb	Average titer $0.8 \pm 0.7 \times 10^9$ pfu/ml
• ceNOS	3.6	
• hTERT	3.5	
• hTERT D712A	3.5	
• lacZ	3.2	
• hCAT1	2.2	
• GLVR2	2.0	
• Luc	1.7	
• SOD3	1.4	
• MAX1	.550	
• hVEGF121	.511	
• hIL3	.434	
• UBC9	.412	
• ANG1-7	.104	

FIGURE 28

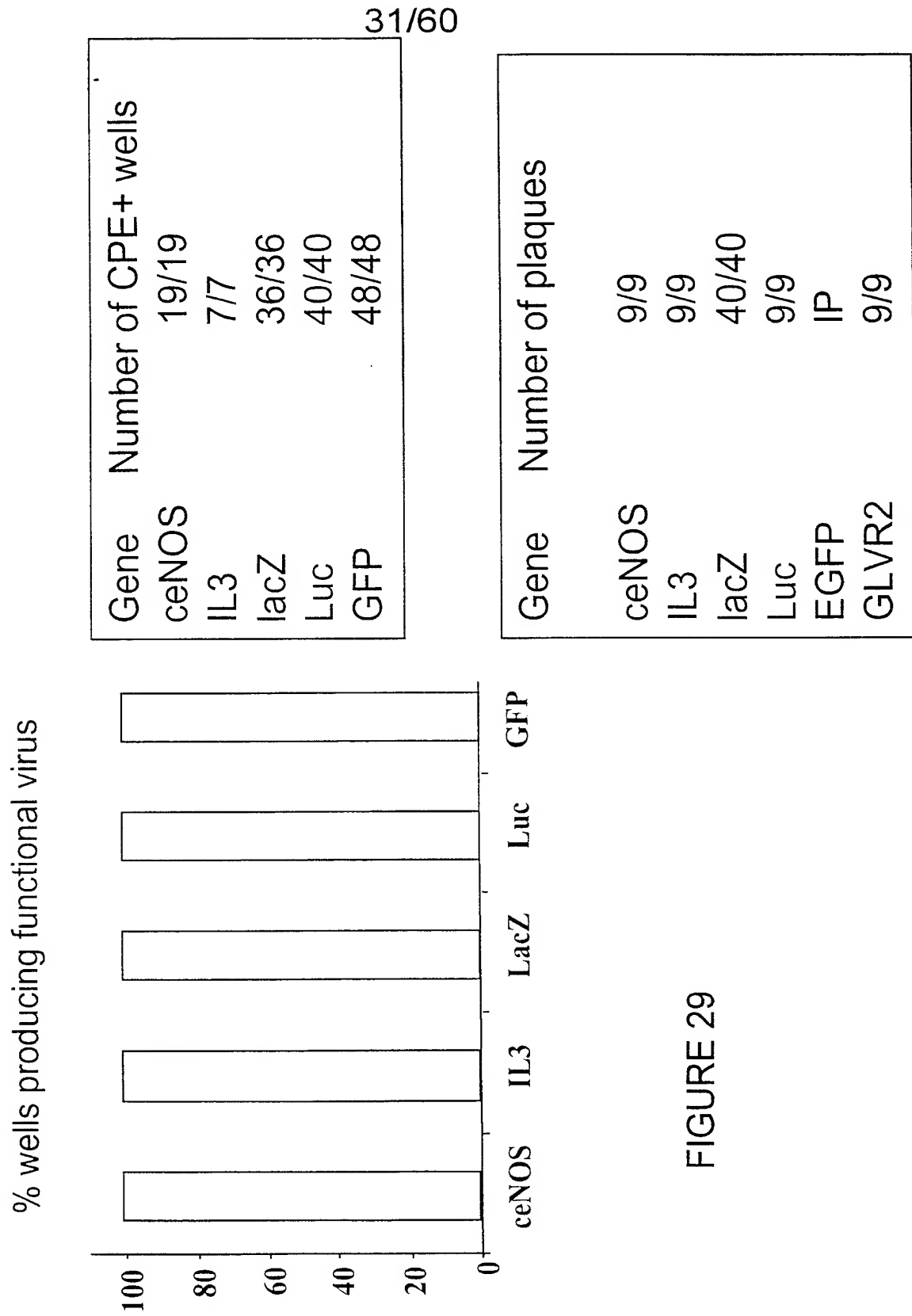
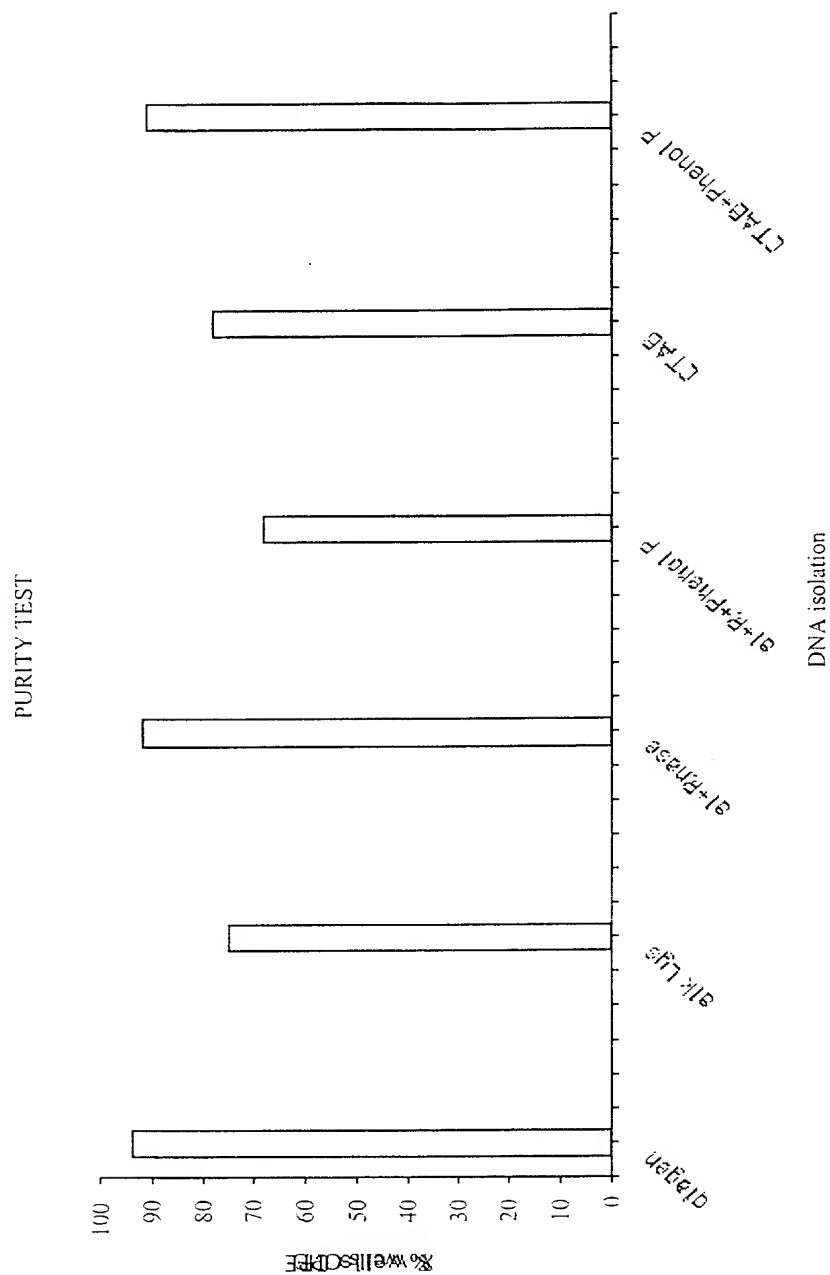


FIGURE 29

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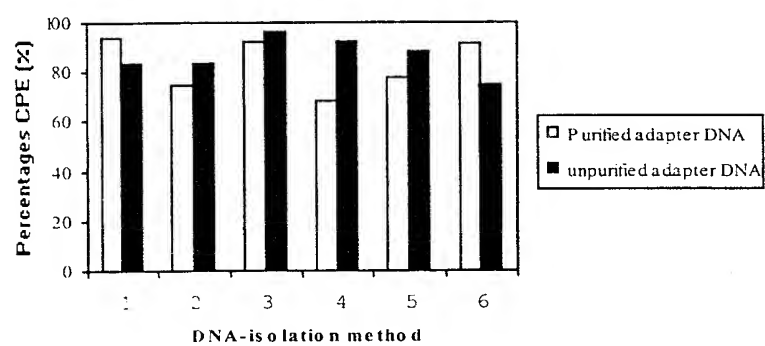
FIGURE 30



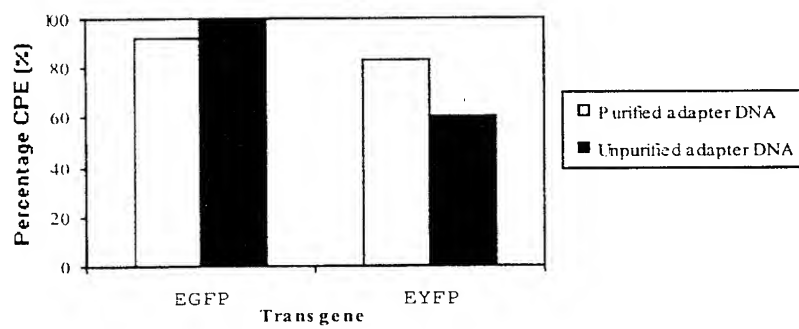
33/60

FIGURE 31

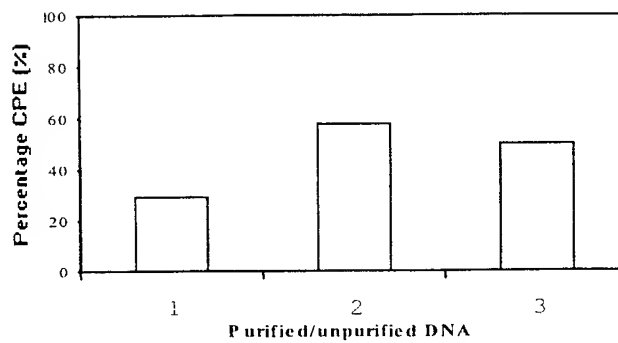
A



B



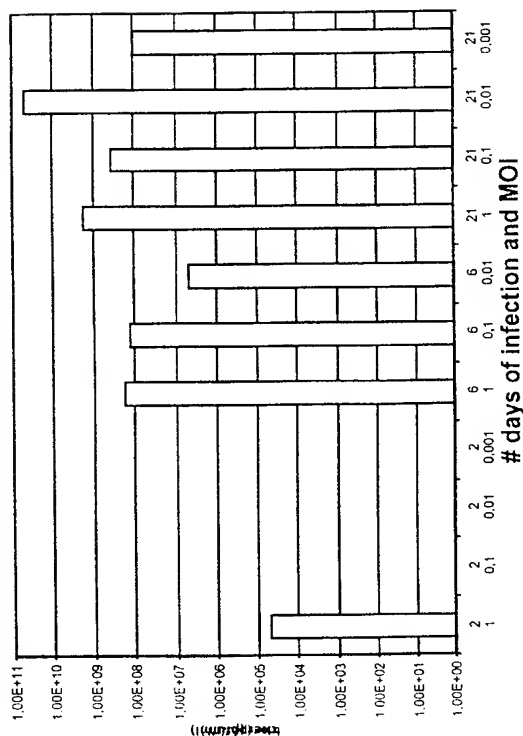
C



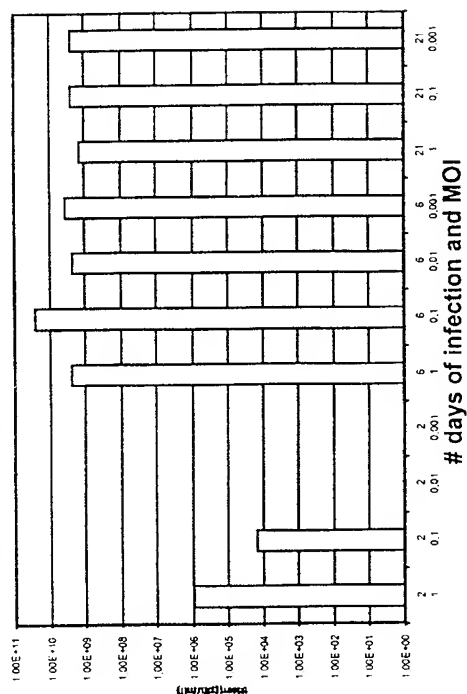


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titer of lacZ adenovector incubated after infection at 32°C



titer of lacZ adenovector incubated after infection at 34°C



titer of lacZ adenovector incubated after infection at 37°C

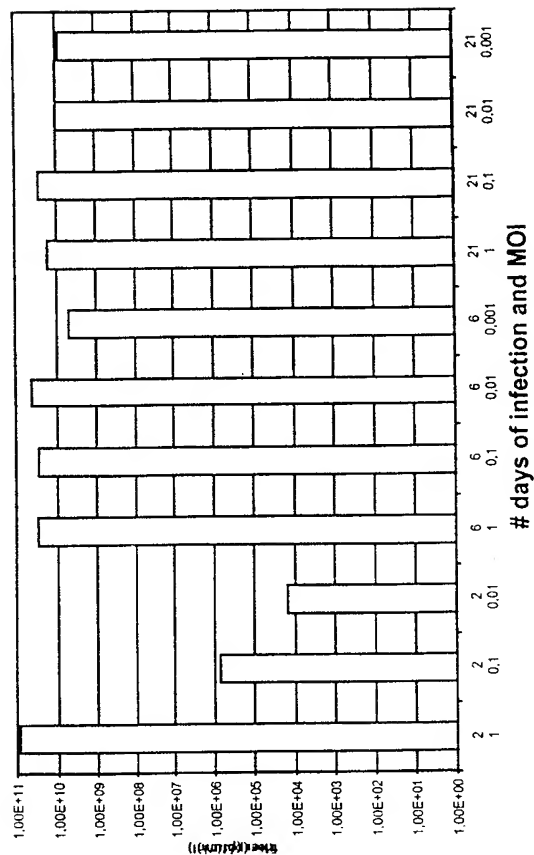
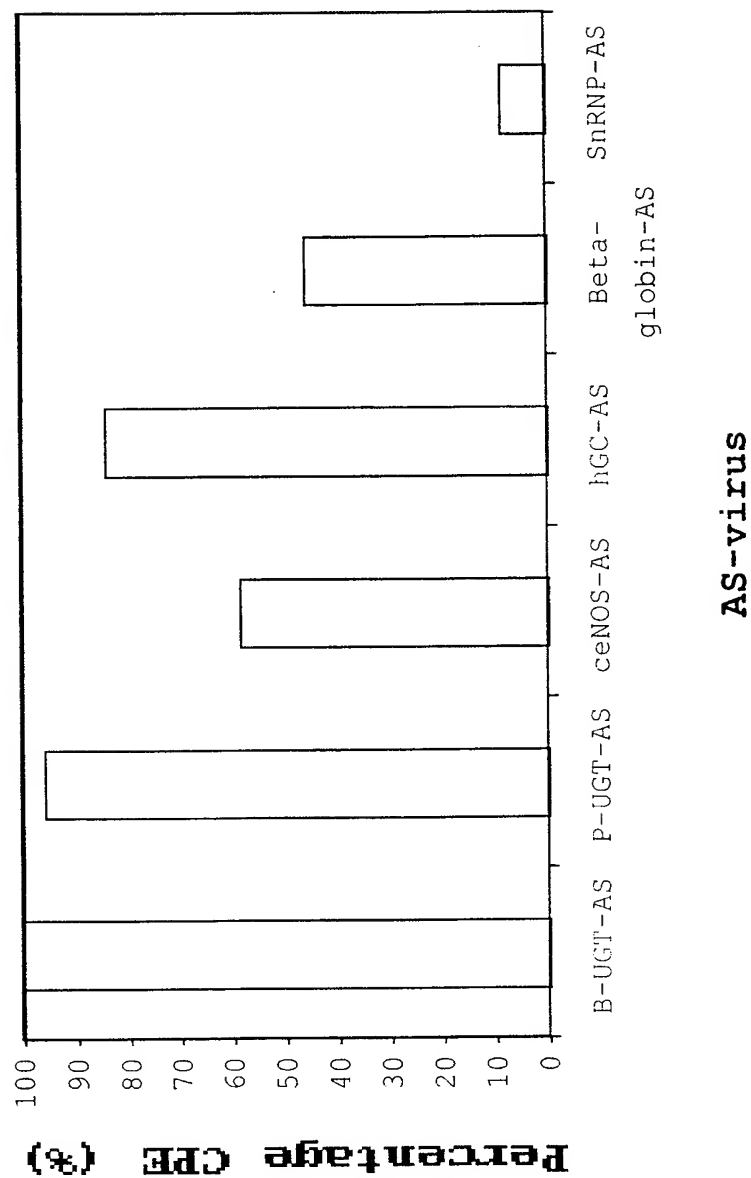


FIGURE 32

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FIGURE 33



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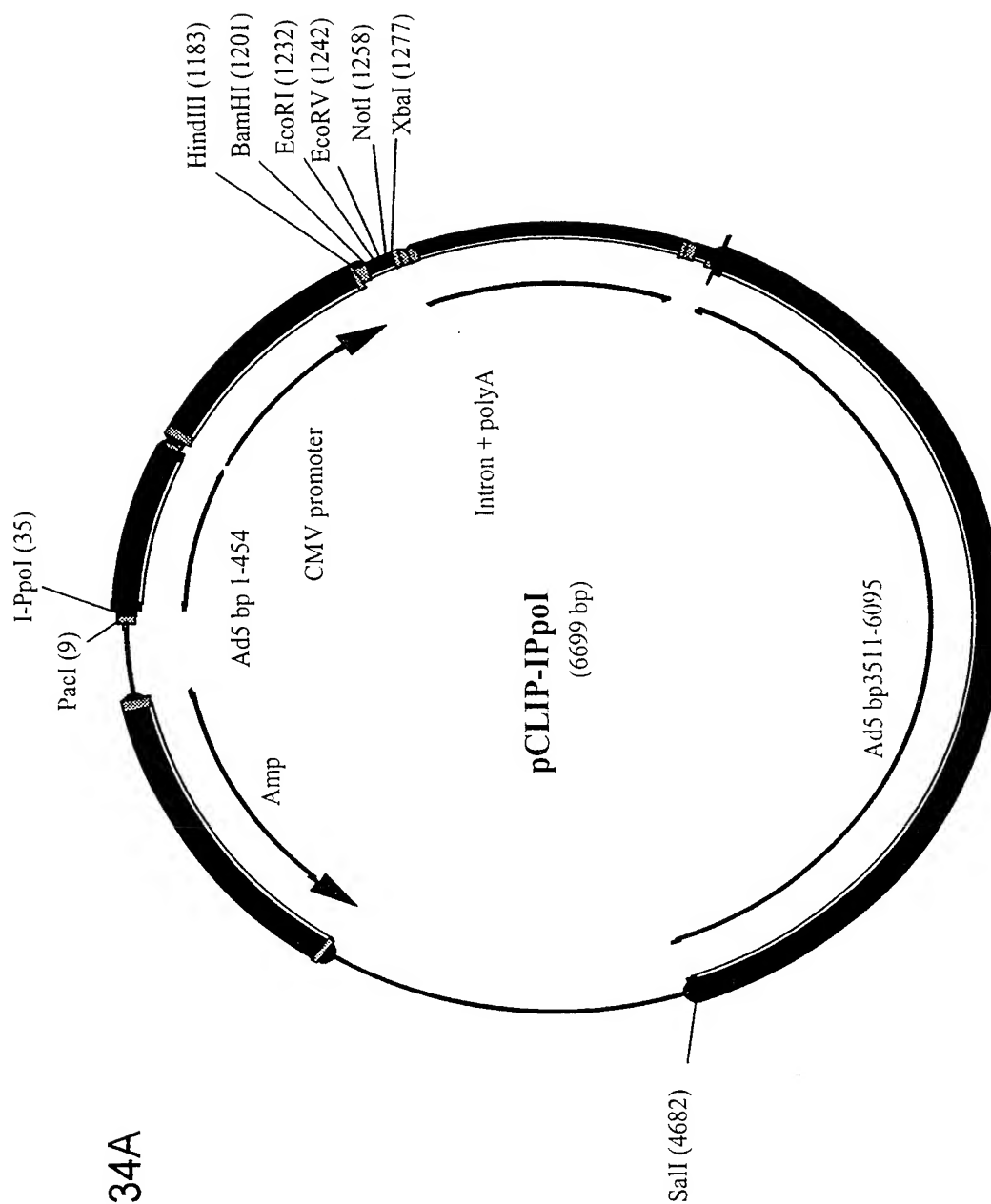


FIGURE 34A

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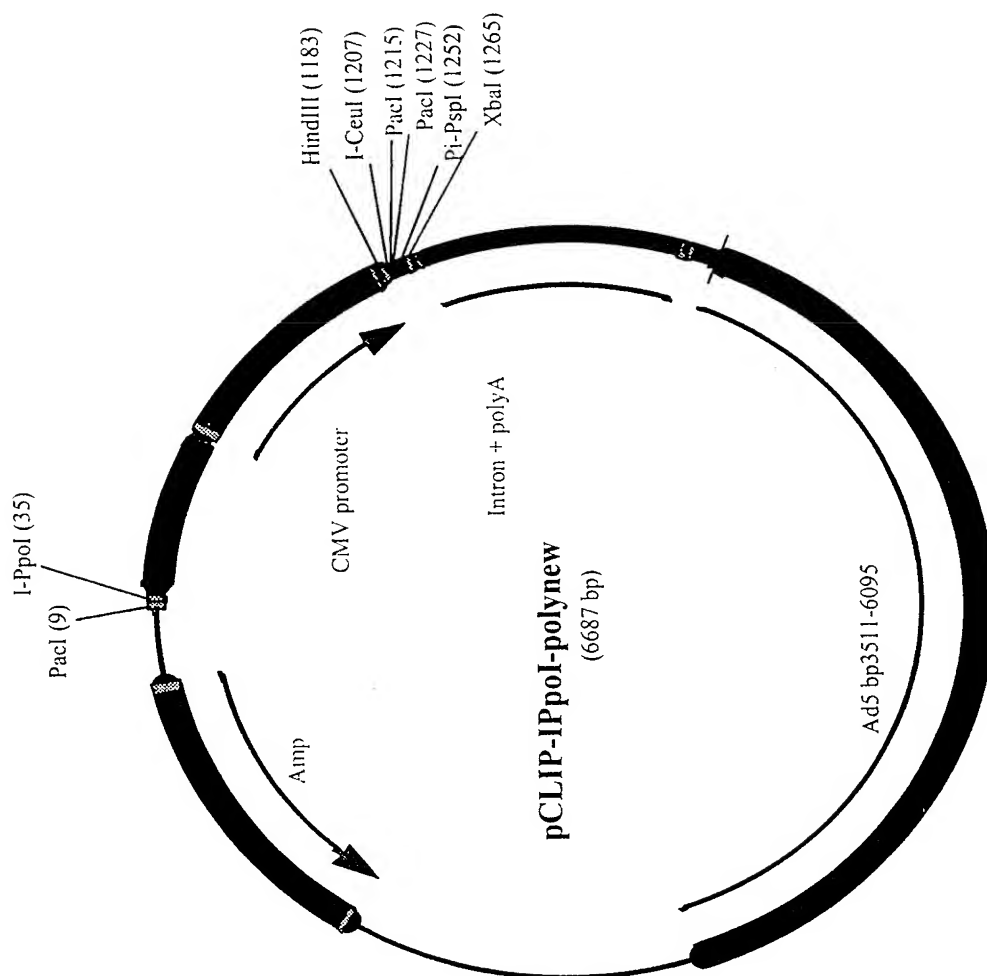


FIGURE 34B

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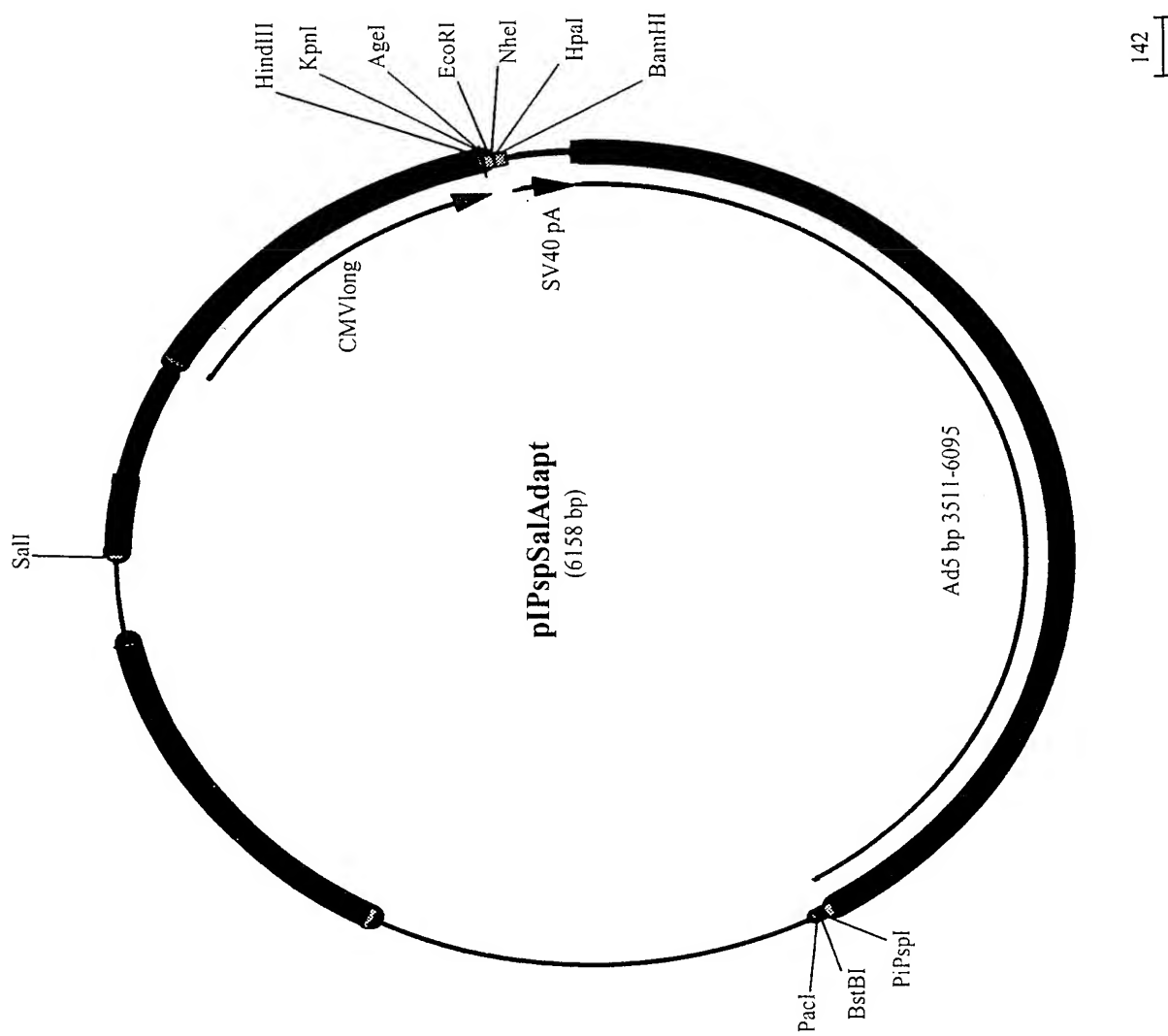
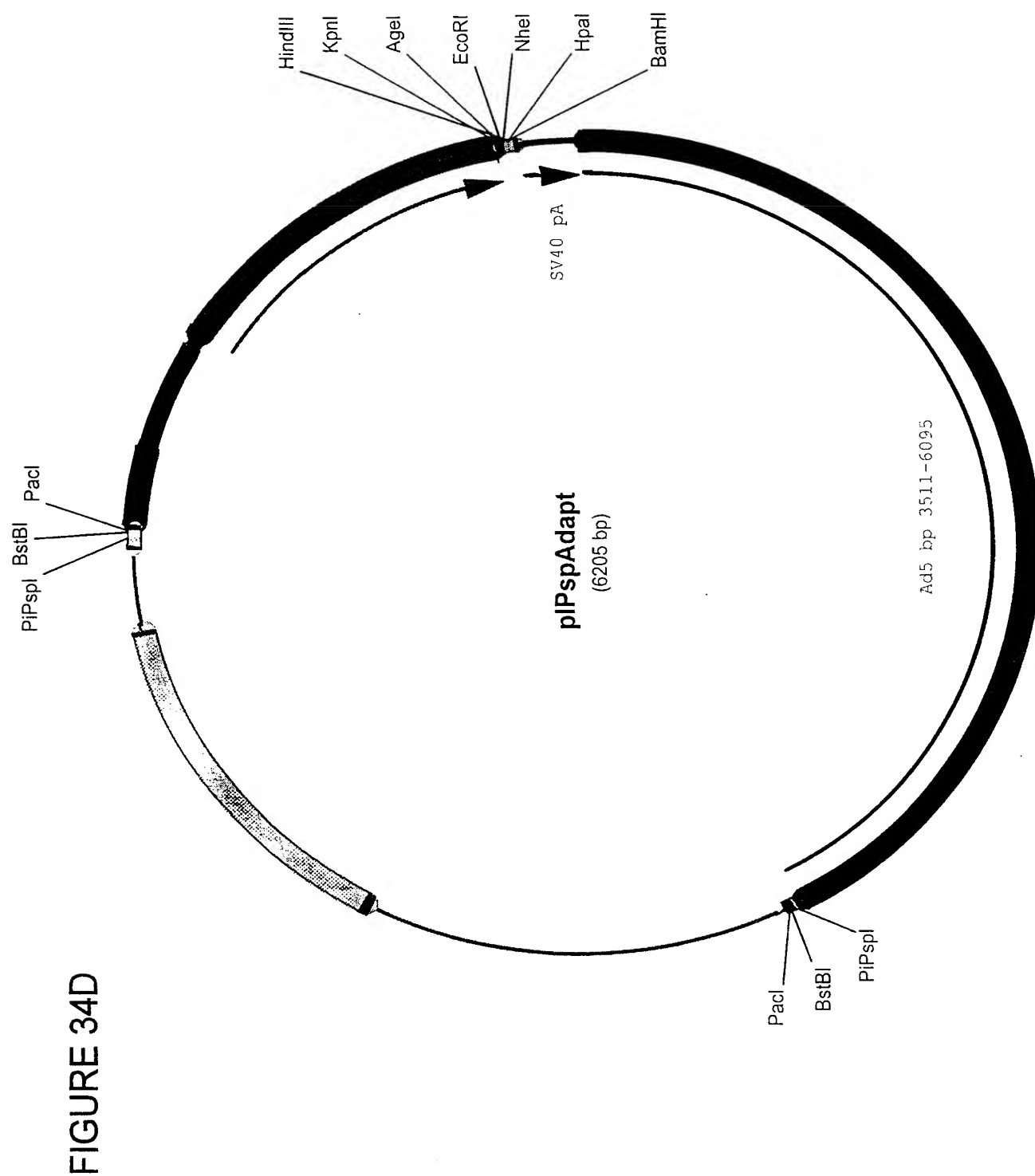


FIGURE 34C

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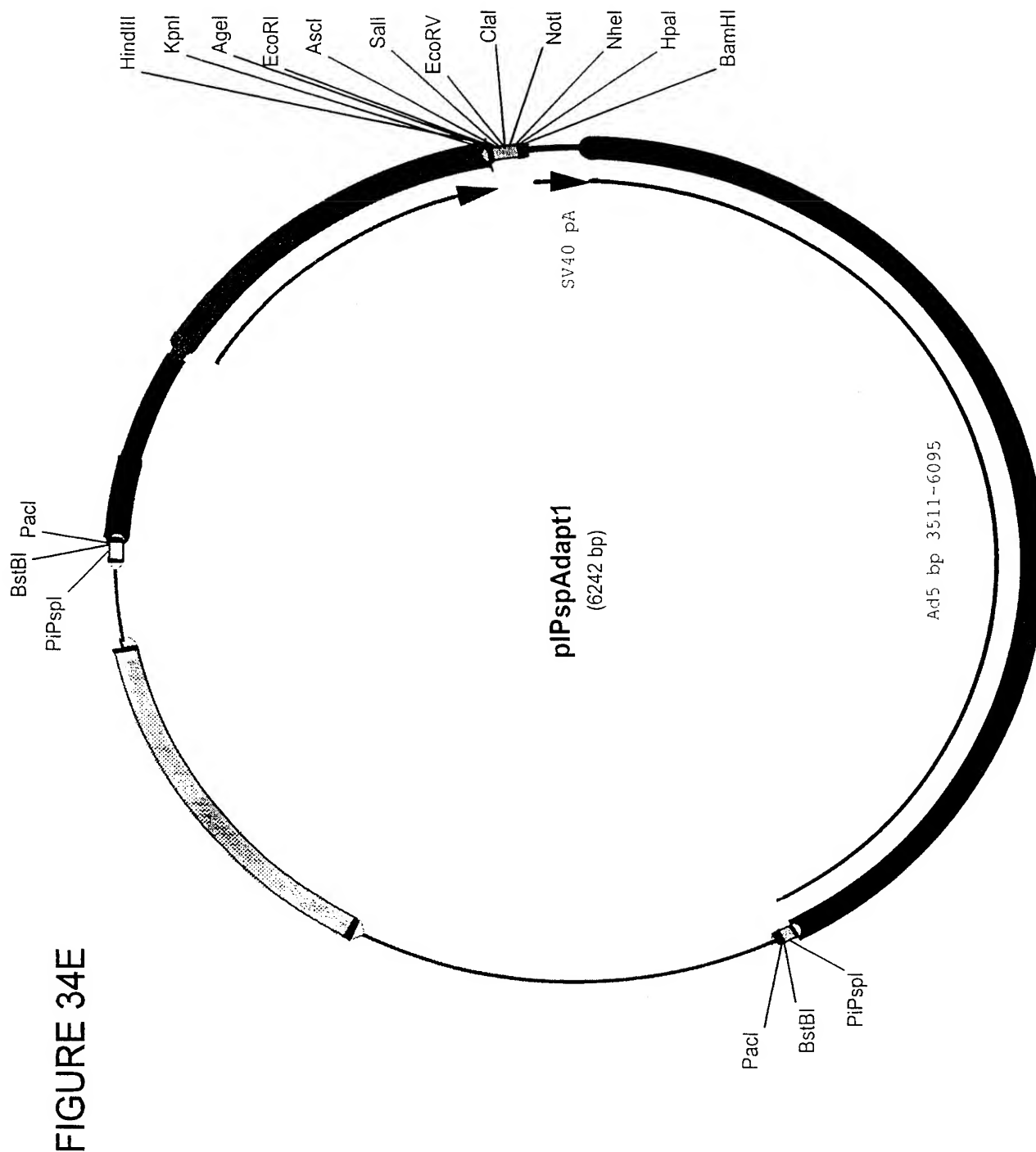


FIGURE 34E

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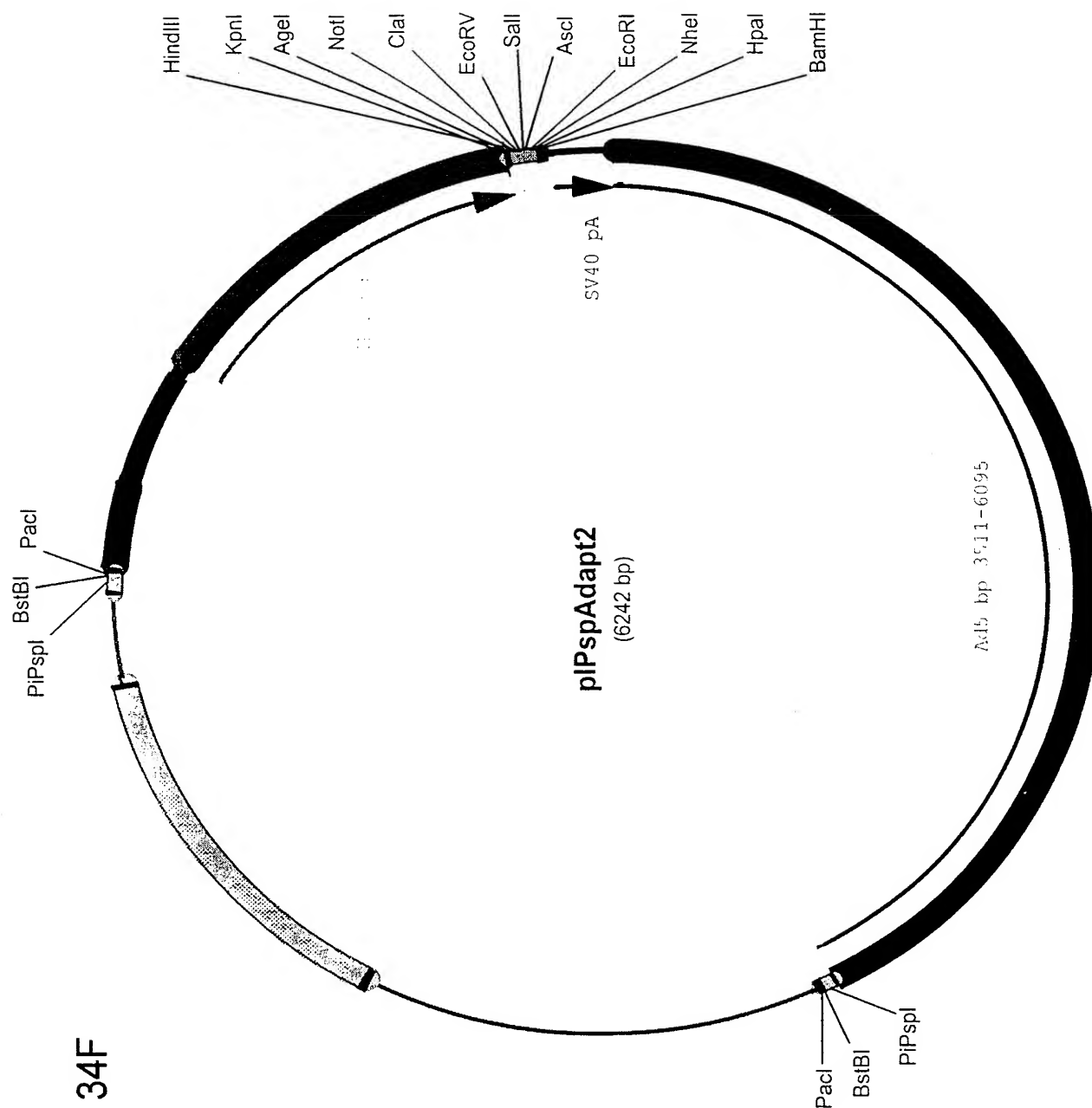
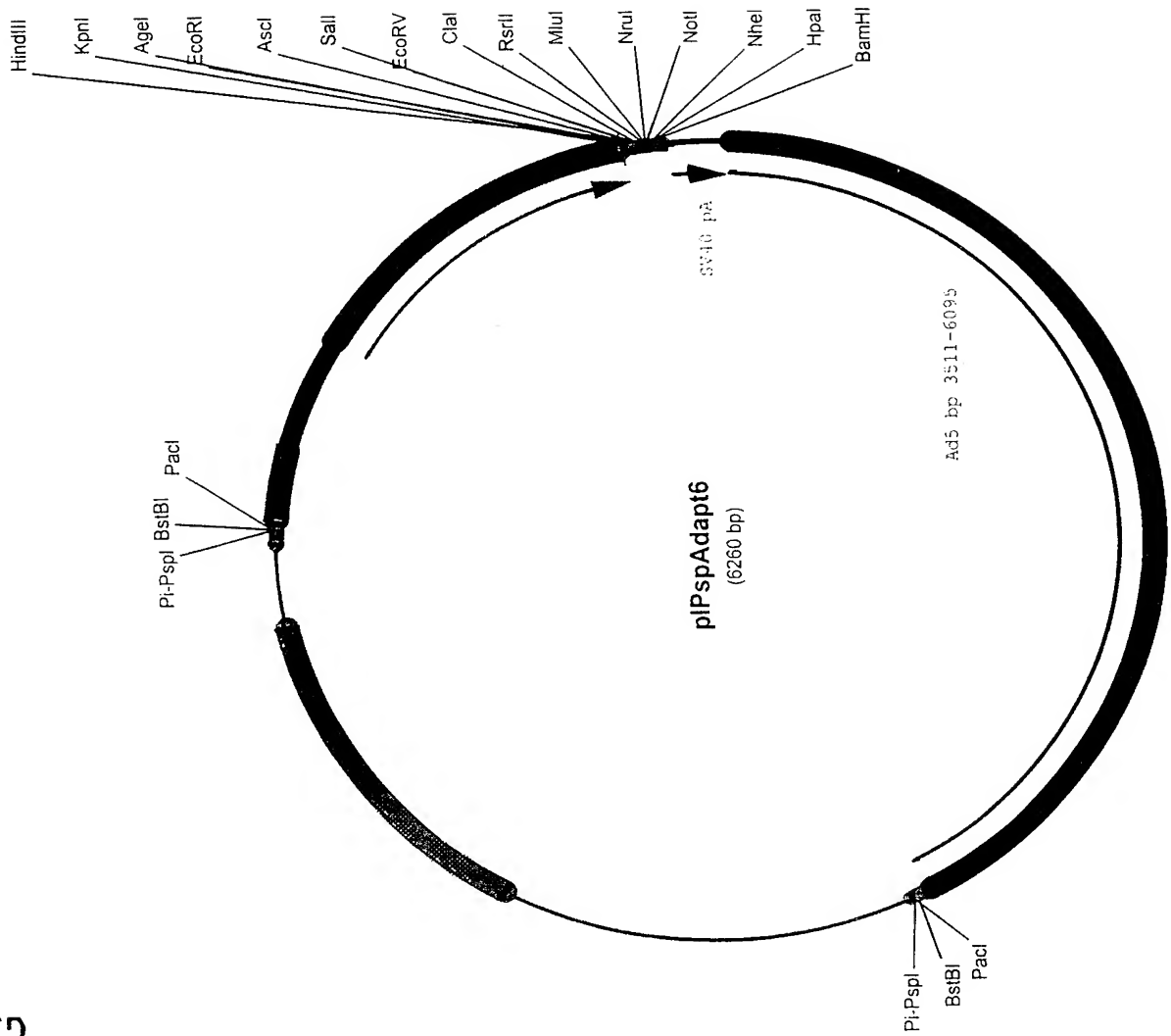


FIGURE 34F



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FIGURE 34G



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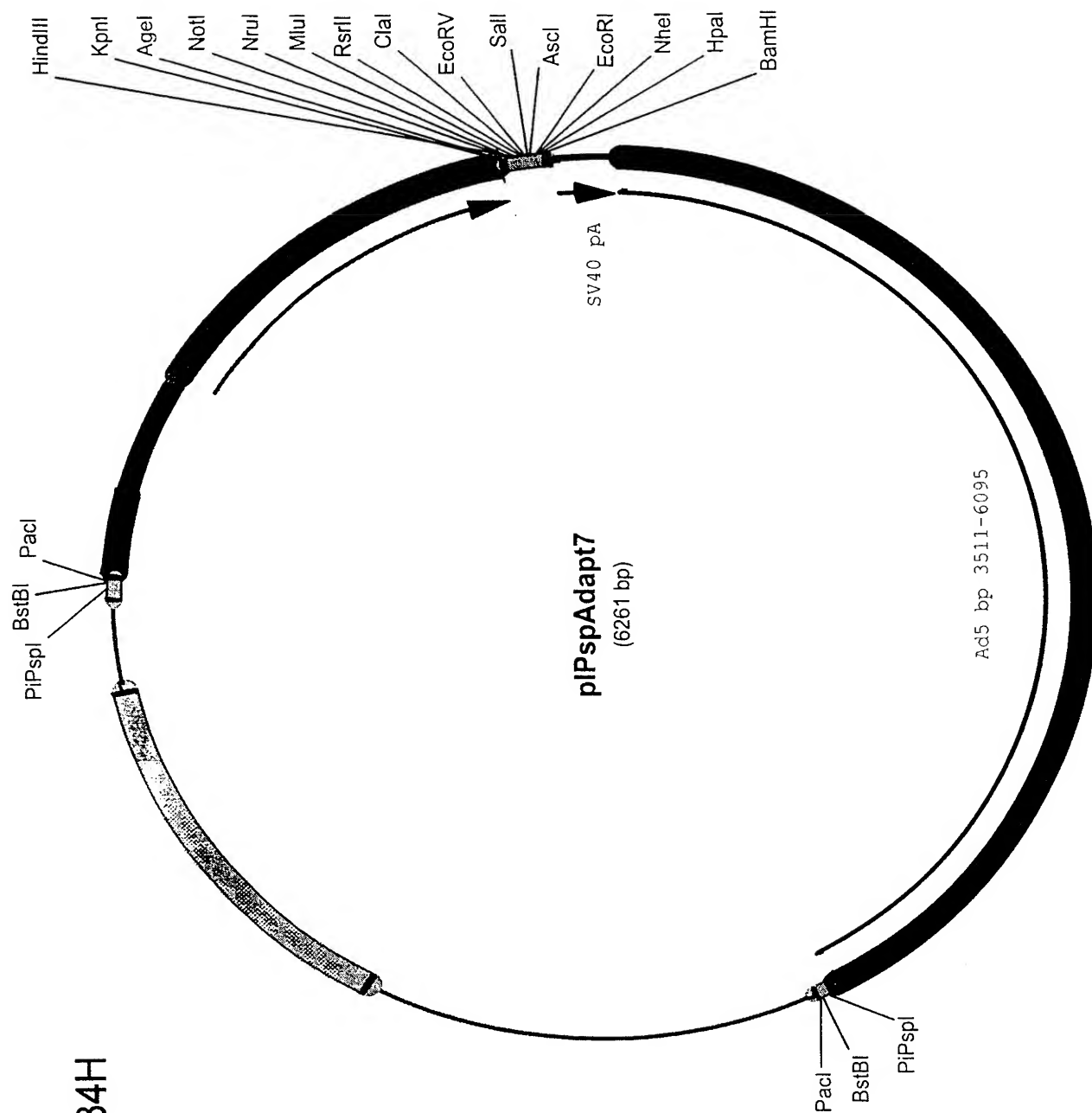


FIGURE 34H

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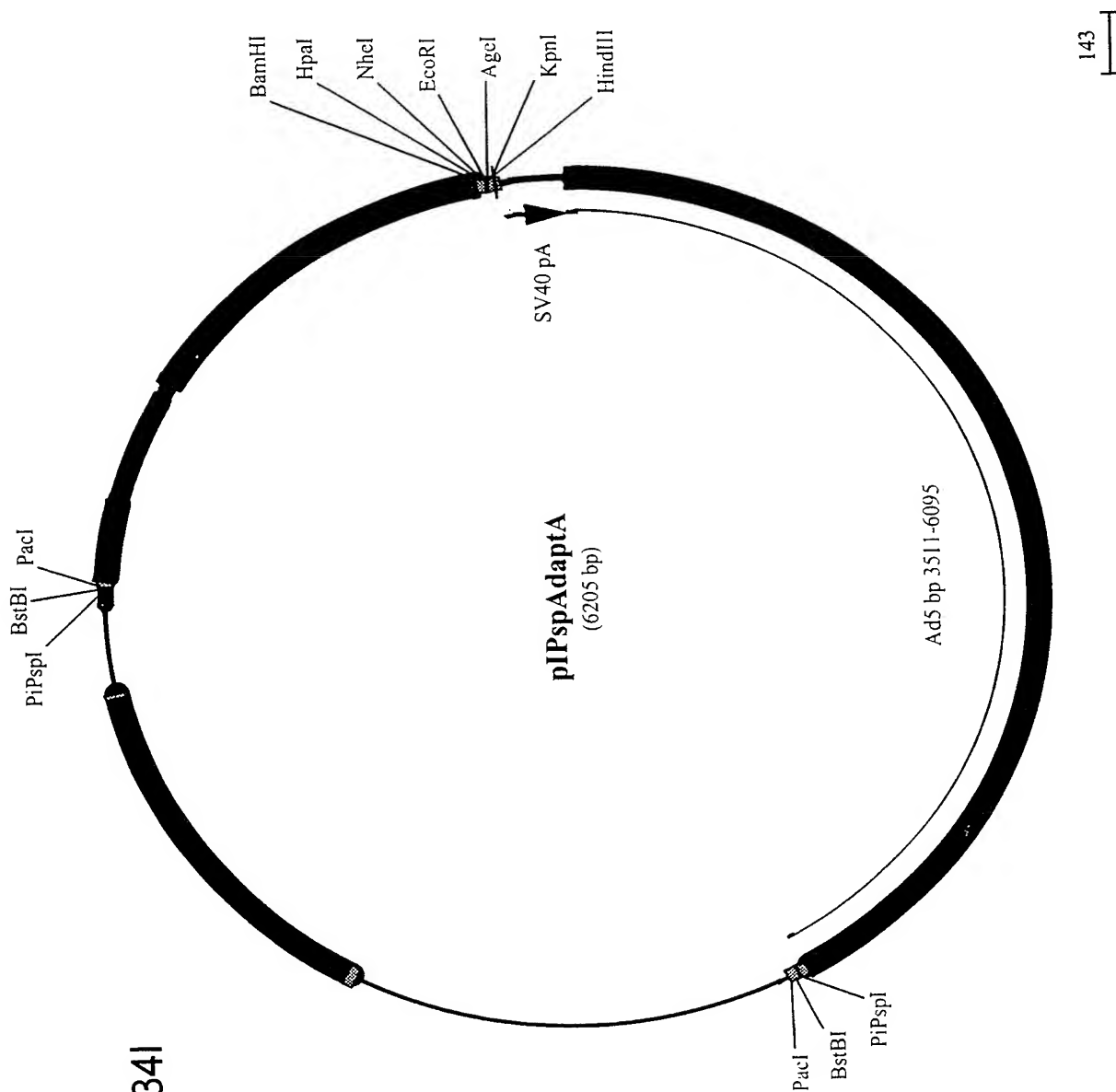


FIGURE 34I

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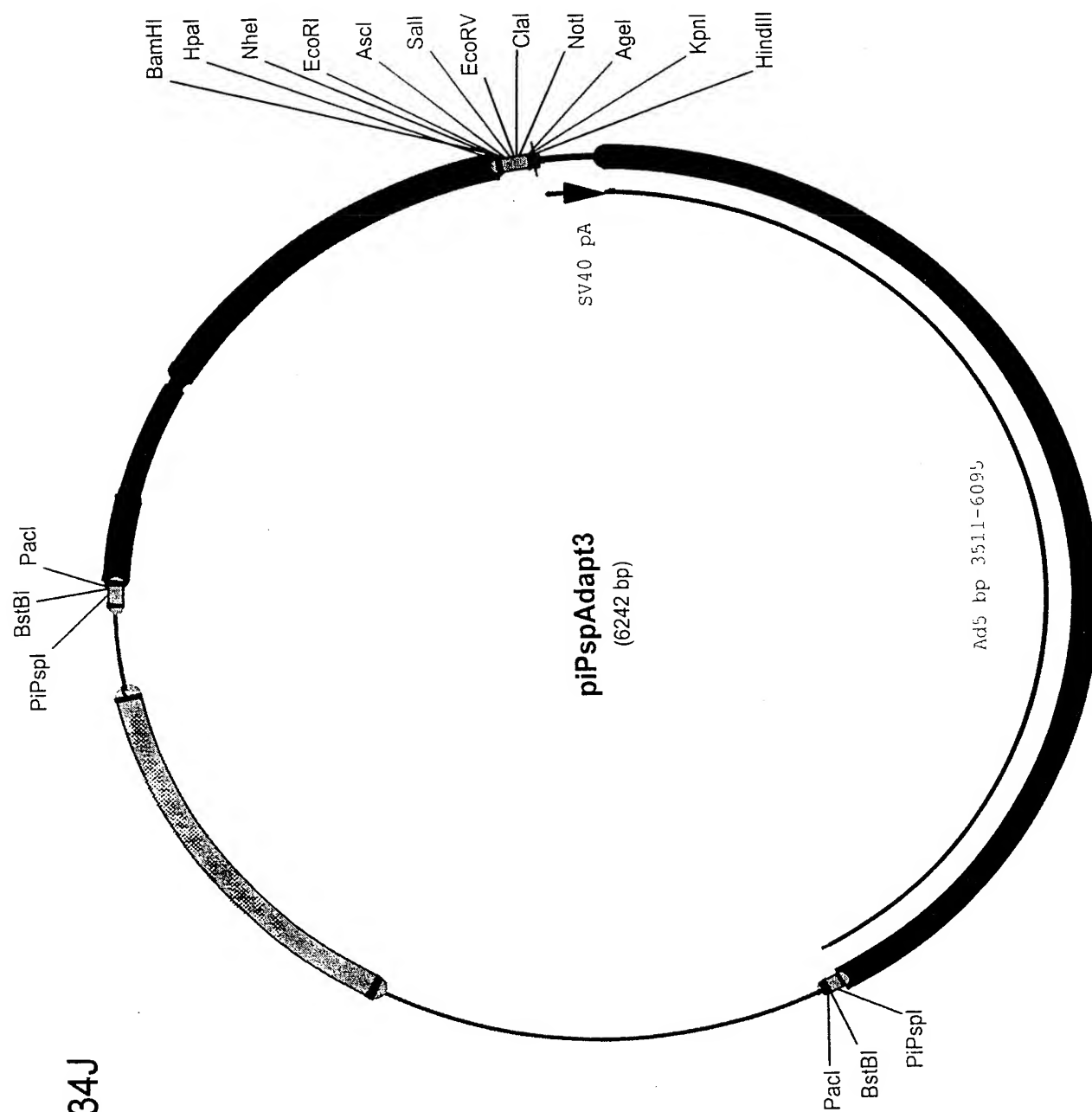


FIGURE 34J

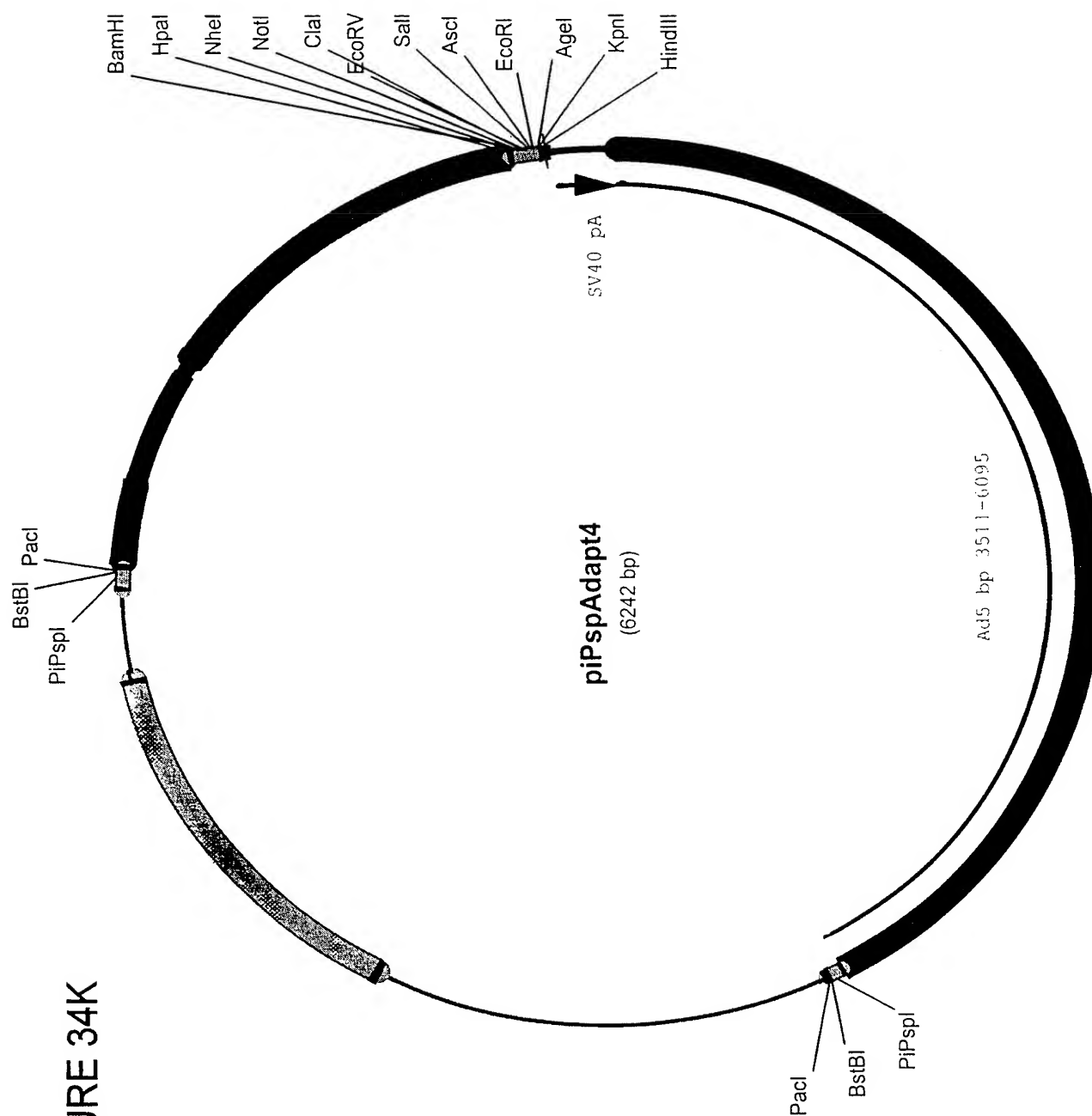


FIGURE 34K

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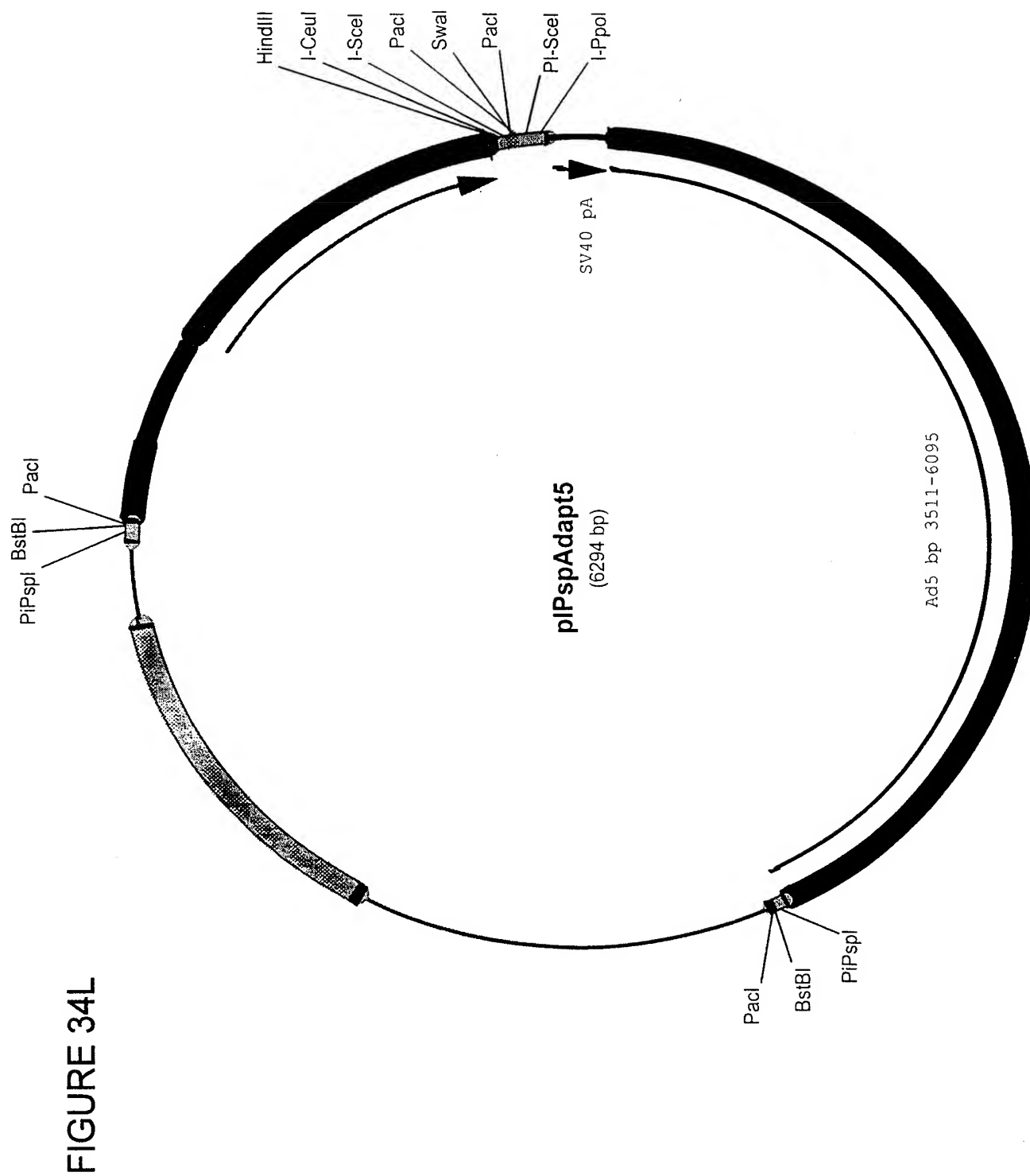
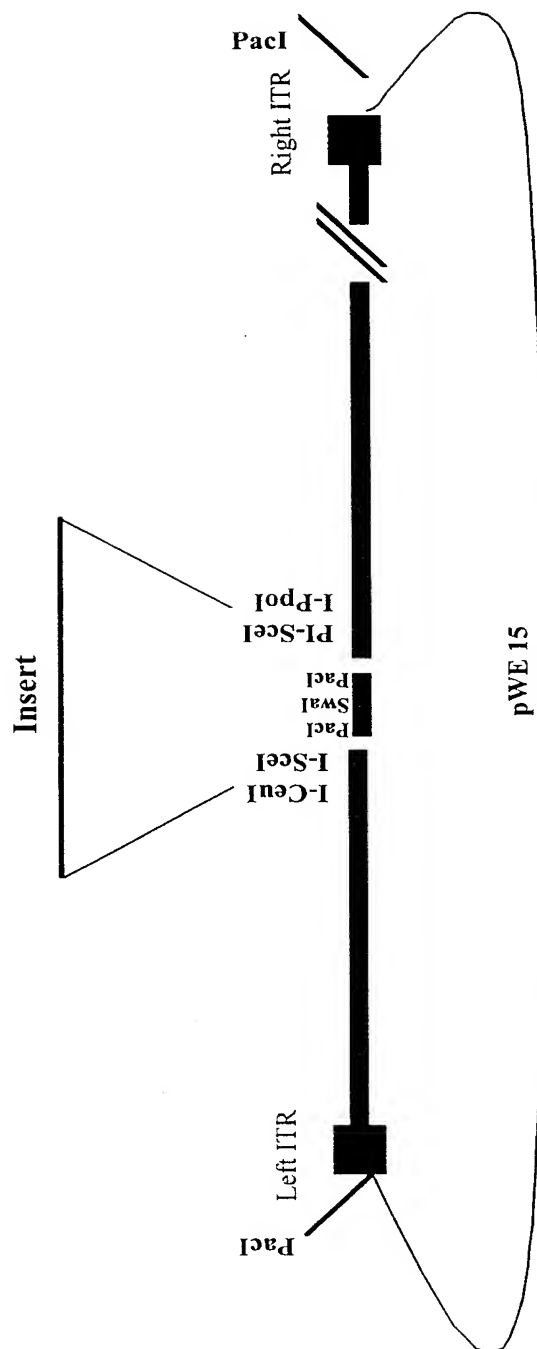


FIGURE 34L

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FIGURE 34M



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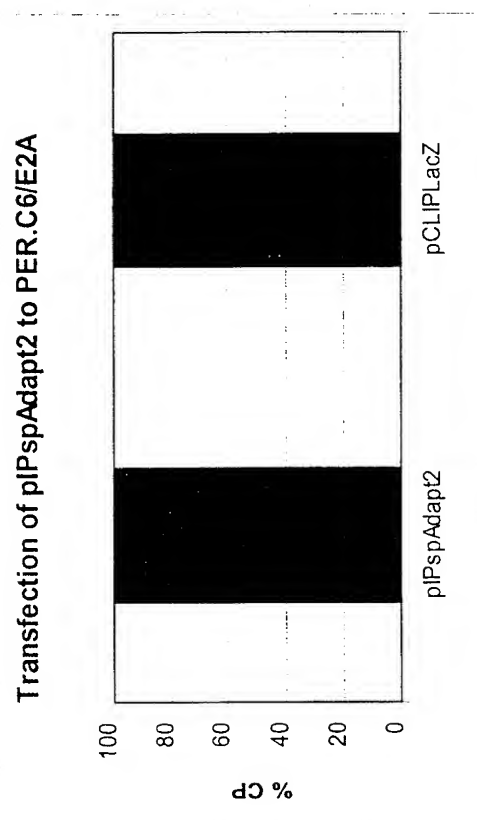


FIGURE 34N. Relative amounts of wells with CPE after transfection of PER.C6/E2A cells with pCLIP-LacZ and the adapter plasmid pIPspAdapt2.



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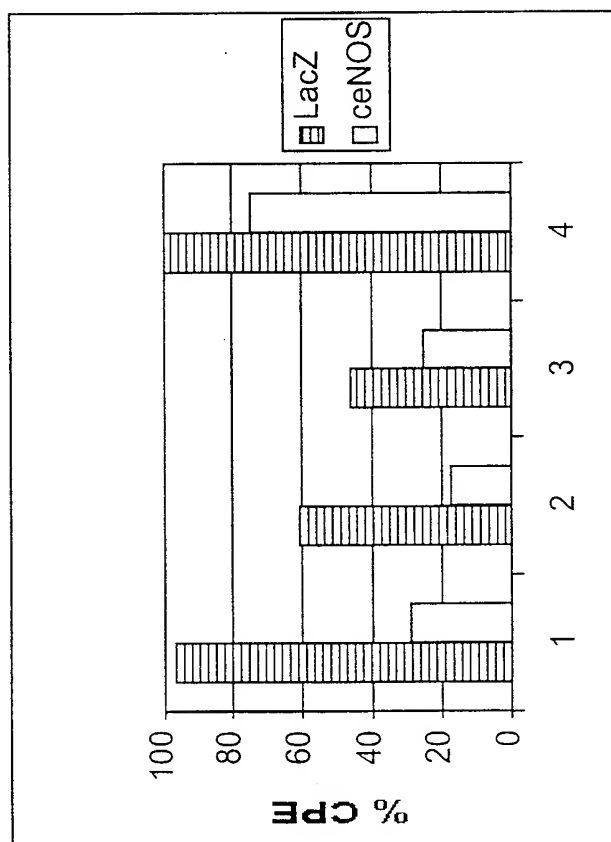


FIGURE 35

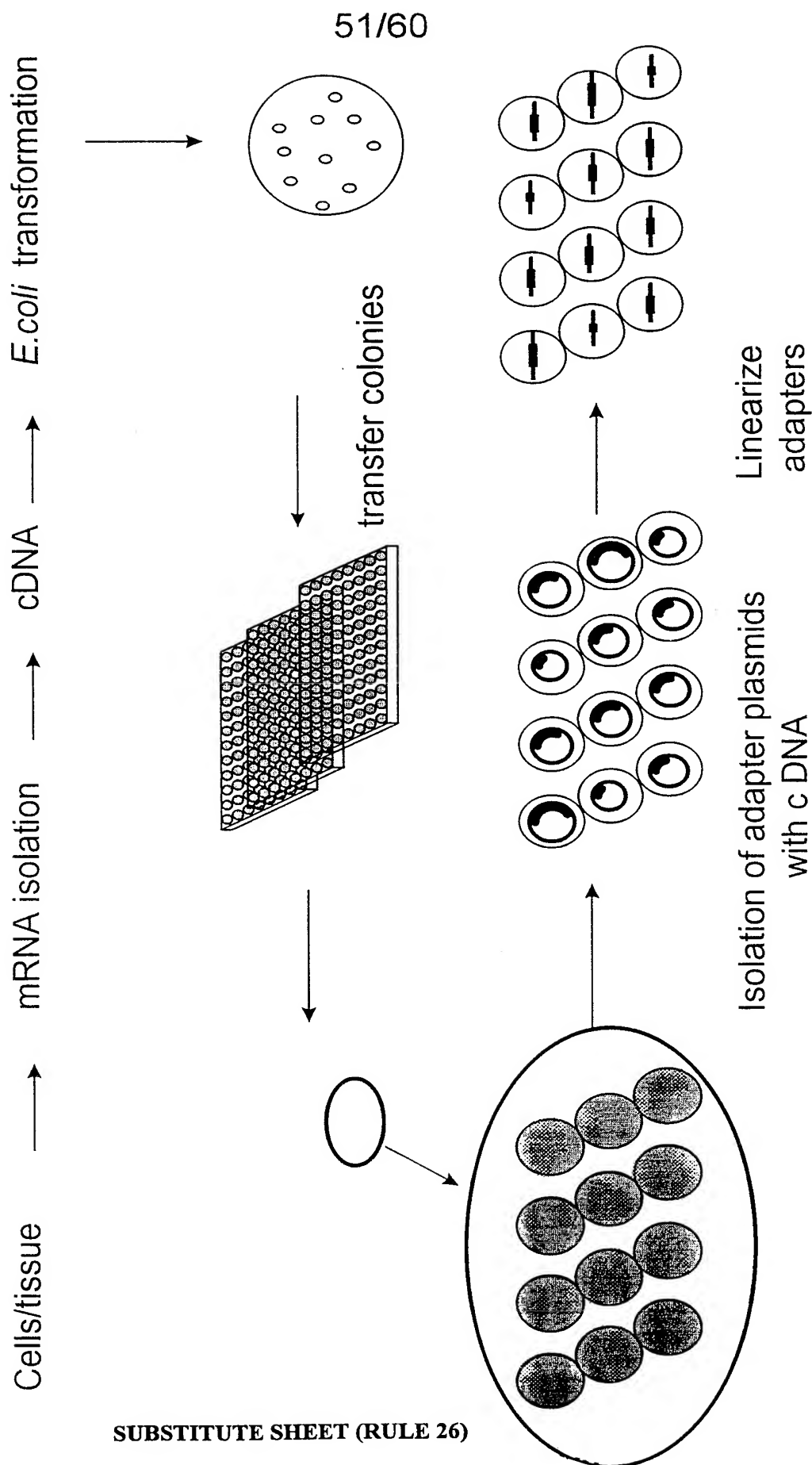


FIGURE 36 Construction total Adeno cDNA Library (I)

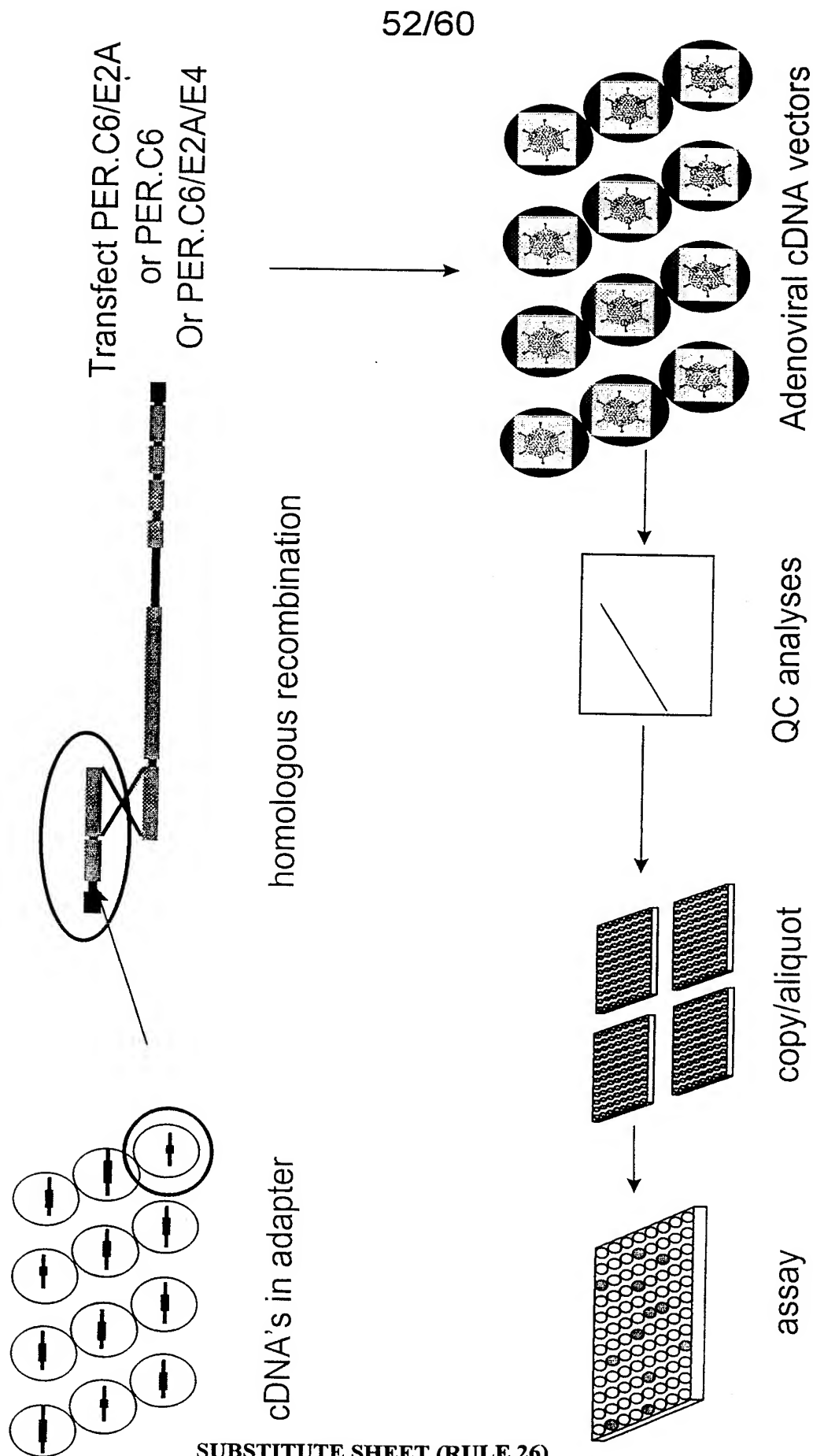


FIGURE 36 Construction total Adeno cDNA Library (II)

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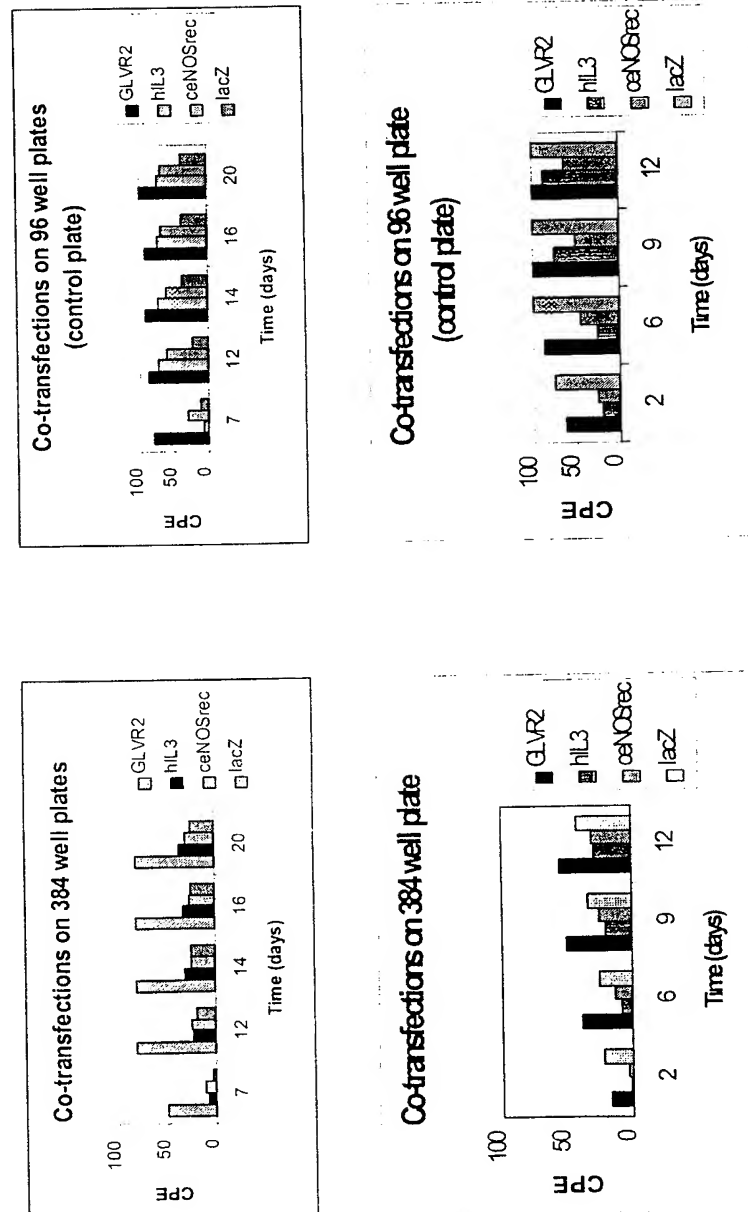
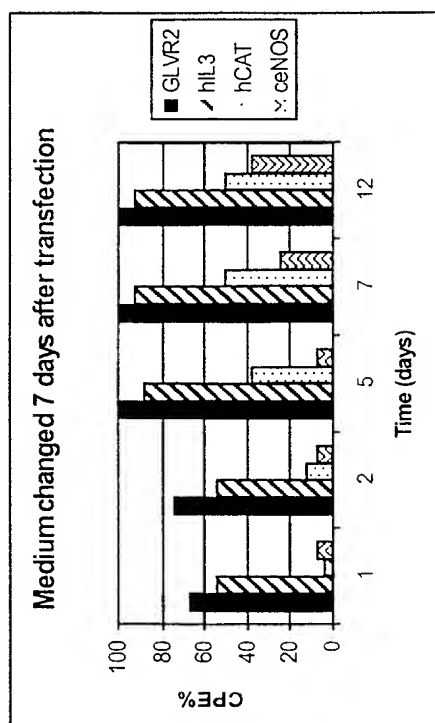


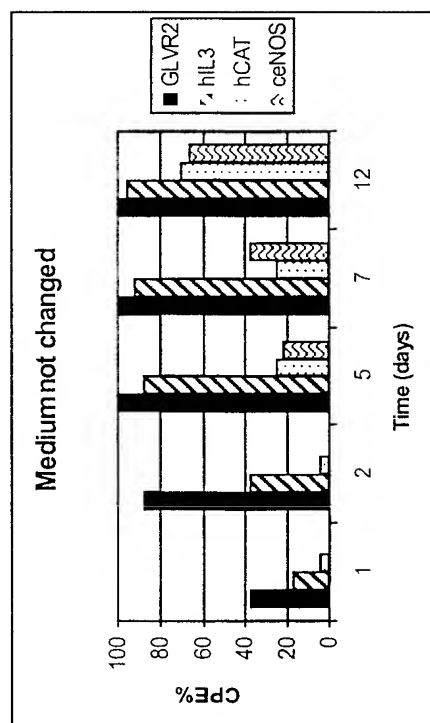
FIGURE 37 EXAMPLE 21 384 WELL PLATE IN PROGRESS

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A



B



C

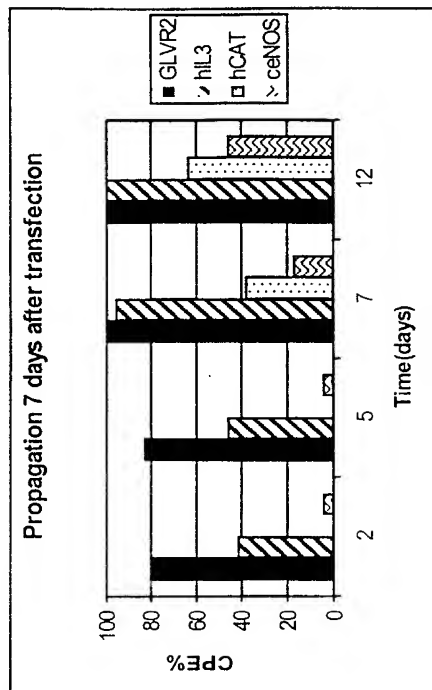


FIGURE 38

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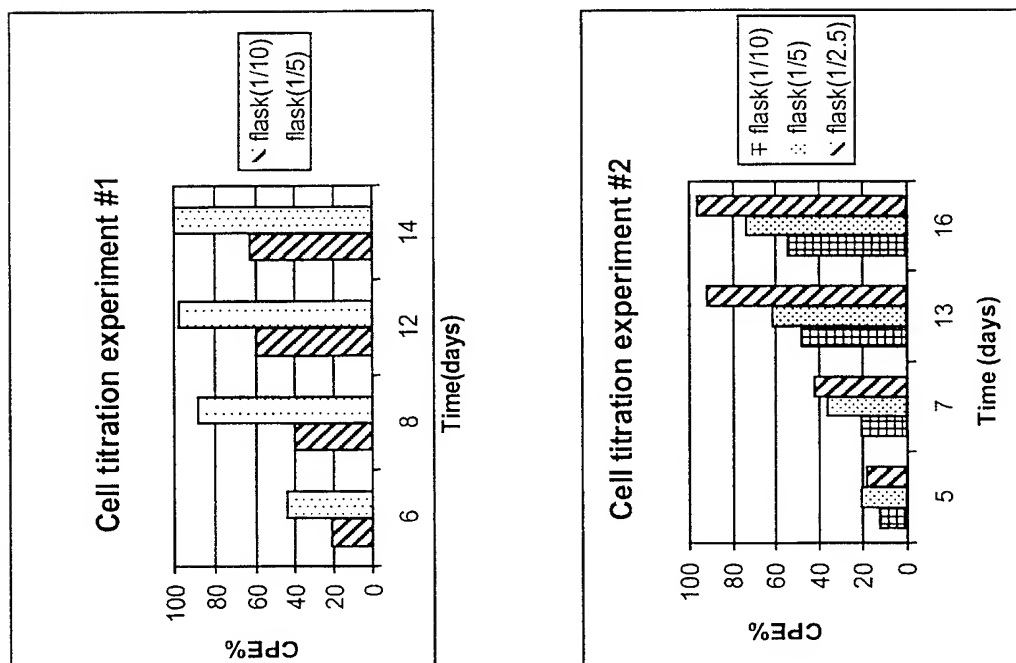


FIGURE 39

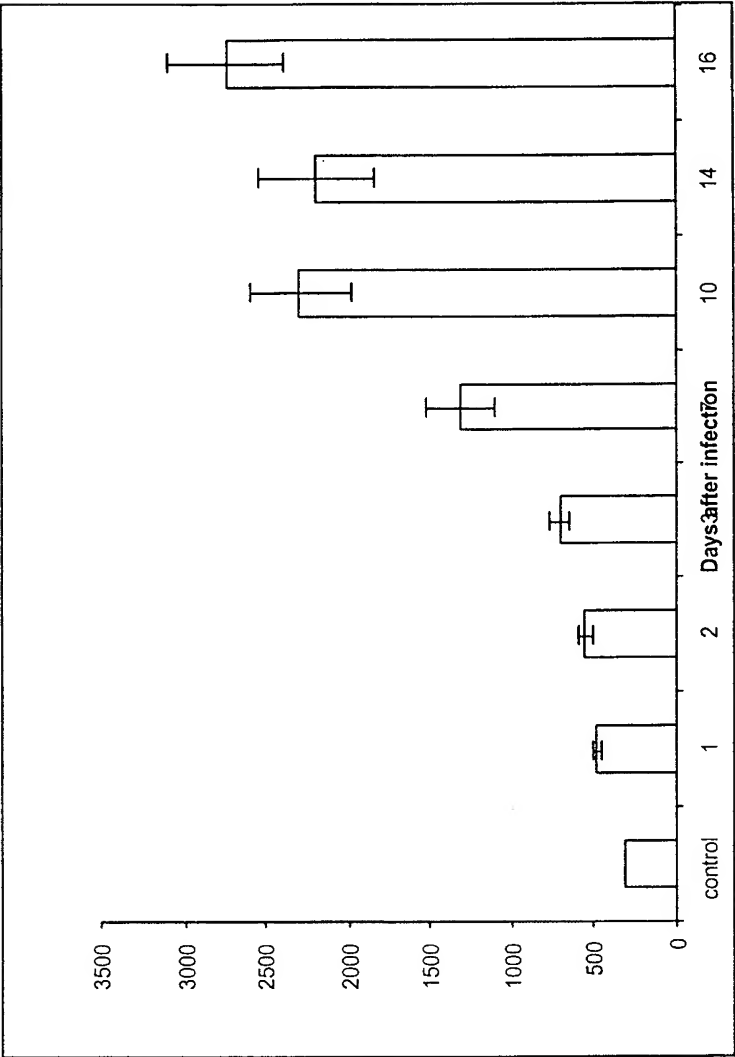
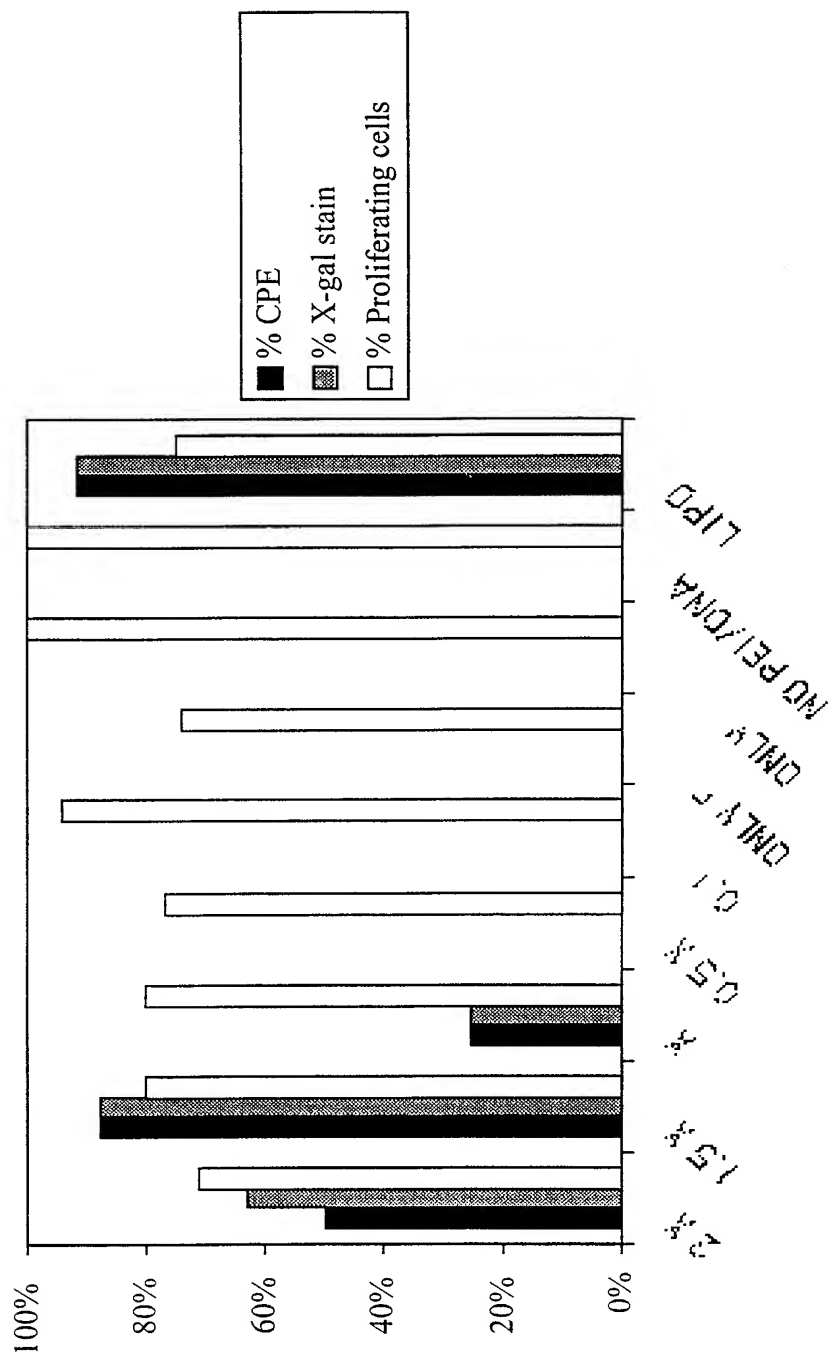


FIGURE 40

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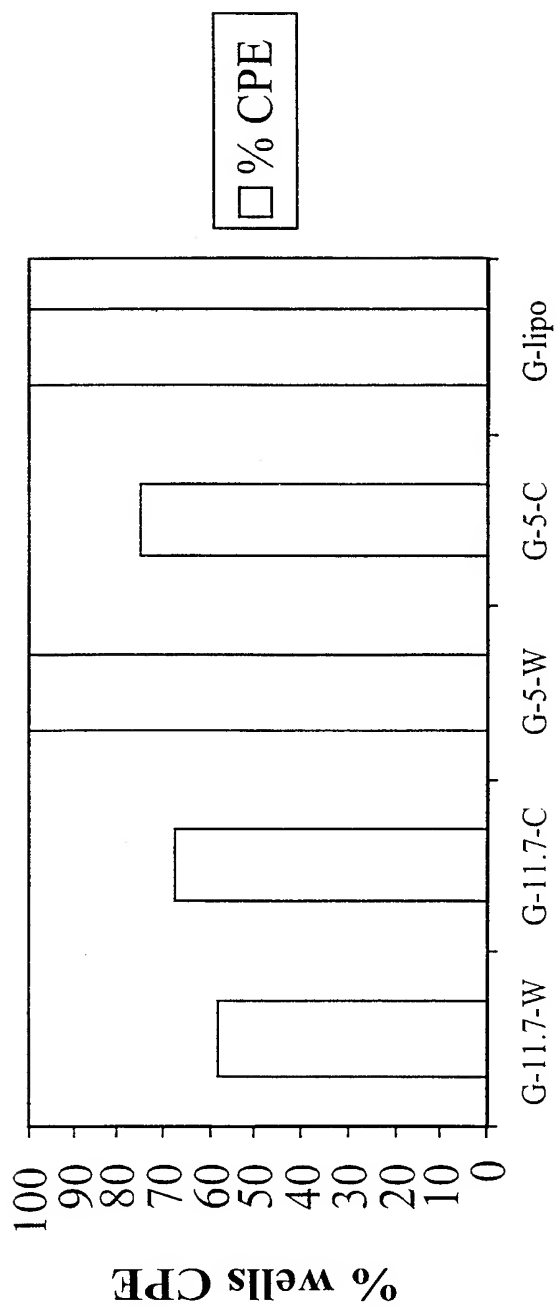
FIGURE 41





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FIGURE 42



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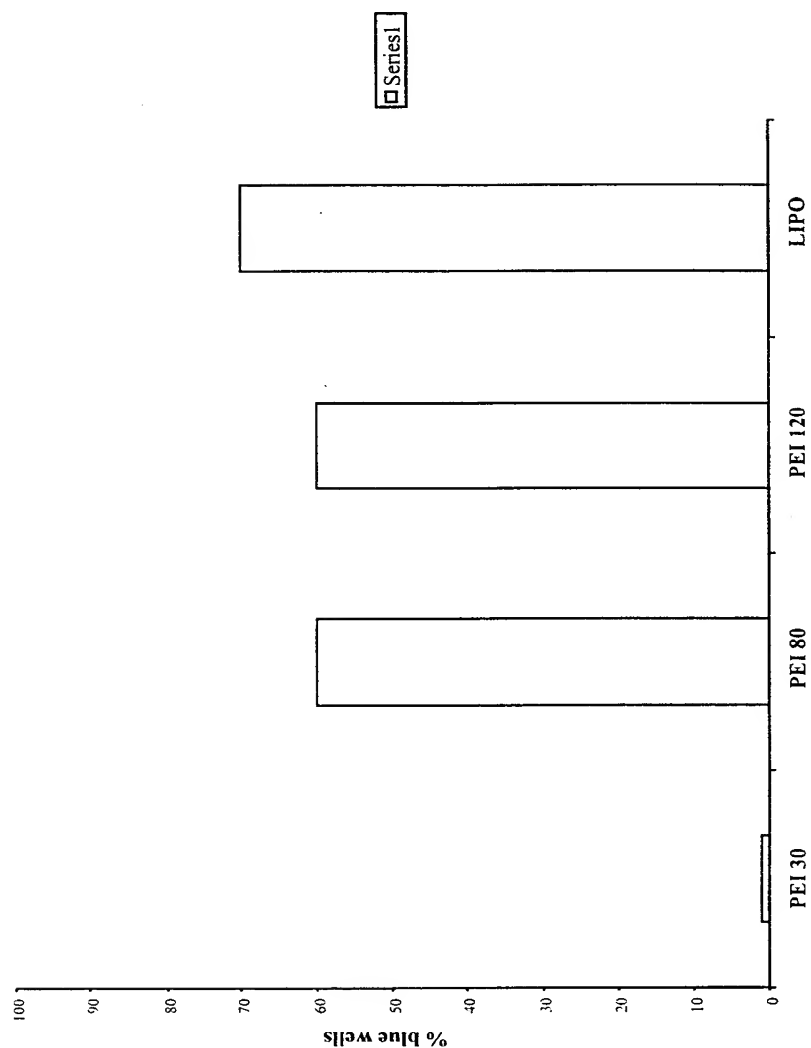


FIGURE 43

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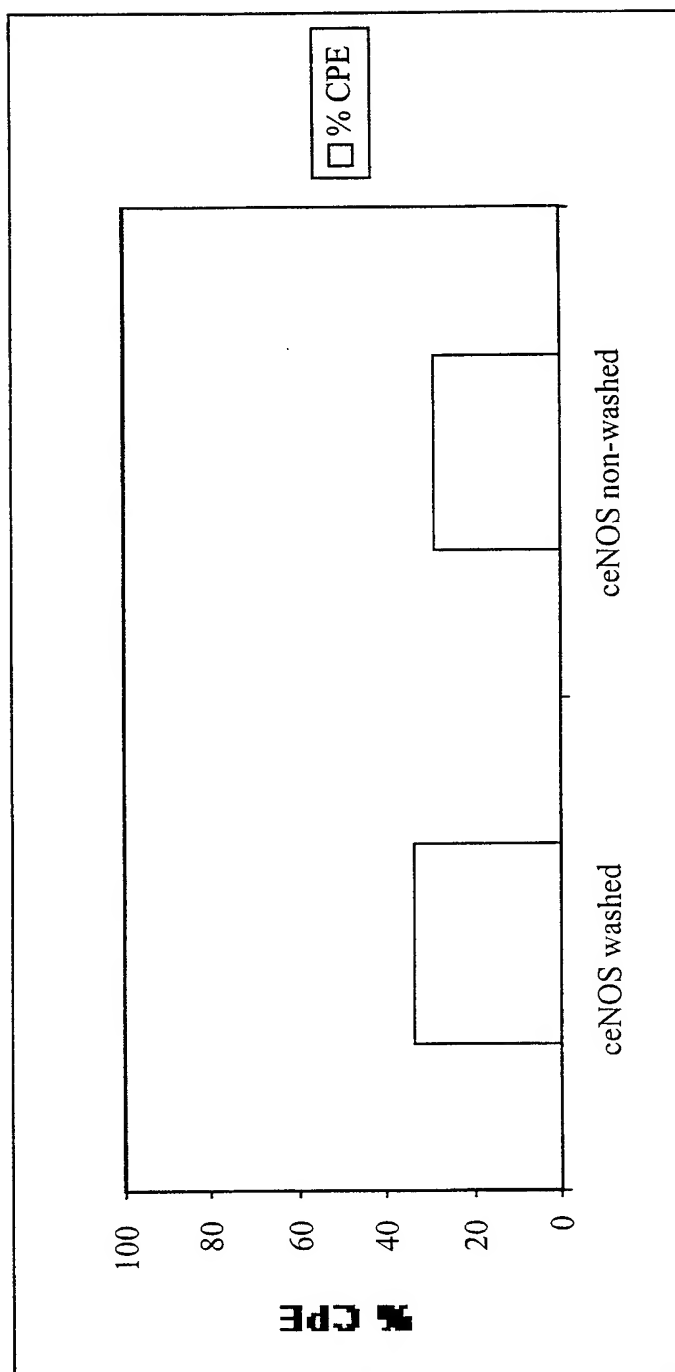


FIGURE 44